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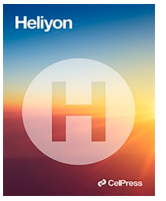

Circulating choline and phosphocholine measurement by a hydrophilic interaction liquid chromatography–tandem mass spectrometry

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ABSTRACT

Background: Given the growing interest in studying the role of choline and phosphocholine in the development and progression of tumor pathology, in this study we describe the development and validation of a fast and robust method for the simultaneous analysis of choline and phosphocholine in human plasma.
Methods: Choline and phosphocholine quantification in human plasma was obtained using a hydrophilic interaction liquid chromatography–tandem mass spectrometry technique. Assay performance parameters were evaluated using EMA guidelines.
Results: Calibration curve ranged from 0.60 to 38.40 μmol/L ($R^2 = 0.999$) and 0.08–5.43 μmol/L ($R^2 = 0.998$) for choline and phosphocholine, respectively. The Limit Of Detection of the method was 0.06 μmol/L for choline and 0.04 μmol/L for phosphocholine. The coefficient of variation range for intra-assay precision is 2.2–4.1 % (choline) and 3.2–15 % (phosphocholine), and the inter-assay precision range is < 1–6.5 % (choline) and 6.2–20 % (phosphocholine). The accuracy of the method was below the ±20 % benchmarks at all the metabolites concentration levels. In-house plasma pool of apparently healthy adults was tested, and a mean concentration of 15.97 μmol/L for Choline and 0.34 μmol/L for Phosphocholine was quantified.
Conclusions: The developed method shows good reliability in quantifying Choline and Phosphocholine in human plasma for clinical purposes.

1. Introduction

Choline (Cho) is an essential nutrient which plays important role in modulating gene expression, cell membrane signaling [1], lipid transport and metabolism [2], methyl-group metabolism [3], and brain development throughout the fetal period [4].

An alteration of choline metabolism is often found in neoplastic disease since the growth of tumor cells requires a remodulation of energy metabolism to support uncontrolled replication (for an extended discussion on choline metabolism see reviews [5,6]). The upregulated choline metabolic pathway characterized by increased phosphocholine (PCho) has been suggested to be one of the

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hallmarks of oncogenesis and tumor progression [7]. Despite the great complexity of the biochemical mechanisms underlying this disease, several studies have revealed that some tumors such as breast [6], brain [7], lung [8], colon [9,10], prostate and ovary tumors [11] had in common the altered concentration of phosphocholine (PCho) and total choline (Cho)-containing compounds related to healthy tissue. In addition, some prospective studies have found a relationship between levels of choline [8,9]and/or its derivatives [10] in the onset of neoplastic disease. These findings suggest that the altered choline metabolism at the cellular level may reflect or be a consequence of a host metabolic disorder already present prior to the development of the cancer disease. Choline is water-soluble due to his positive charge, and it is easily diffusible in the extracellular space. Cho is actively transported into the cell, and his cellular uptake mechanism includes Na + -dependent high affinity transport, Na + -independent low-affinity mechanism and low affinity facilitated diffusion [11,12].

Anyhow, increased plasma levels of choline and phosphocholine could be markers of the onset or progression of oncological disease, and their measurement could be a useful tool to assist early diagnosis [13].

Currently, different analytical method of choline detection in human plasma are available by nuclear magnetic resonance (NMR) [14], enzymatic assays based on choline oxidation by choline oxidase [15], and HPLC-MS [16–19].

Nevertheless, there are only few methods developed for the quantitative measurement of both Cho and PCho, in the same analytical run.

Cho and PCho are small polar metabolites with a positively charged moiety (a quaternary amine). The hydrophilic interaction liquid chromatography (HILIC) can be a suitable separation method. HILIC columns are known to have great performances on small and highly polar molecule in different matrices, such as food [20], water, human biofluids and human tissue extracts [21,22] and provide higher retention of polar analytes compared to Reversed-Phase Liquid Chromatography (RPLC). HILIC is based on the organic-rich mobile phase and the water enriched layer partially immobilized on the stationary phase partition equilibrium. Highly polar metabolites are mainly solubilized into the water layer and strongly retained on the column as they have strong electrostatic interaction and hydrogen bonds with the stationary phase [23].

Considering the growing interest in studying the role of Cho and PCho in the onset and progression of tumor pathology, in this study we describe the development and validation of a fast and robust method for the simultaneous analysis of the two cholines in human plasma using HILIC separation coupled to MS/MS technique.

2. Method and materials

2.1. Chemicals and reagents

Choline chloride, phosphocholine chloride calcium salt standards and deuterated internal standards Choline chloride (trimethyl-d9), phosphocholine-d9 chloride calcium salt (tetrahydrate) were purchased from Spectra 2000 s.r.l. (Roma, Italy), ammonium bicarbonate (NH_4HCO_3), Acetonitrile (ACN) and methanol (MeOH) UHPLC grade were purchased from Carlo Erba (Milano, Italy). Bidistilled water was obtained by PureLab Ultra system from ELGA LabWater (High Wycombe, United Kingdom).

2.2. Solutions preparation

Stock solutions of deuterated internal standards (I.S.) choline-d9 and phosphocholine-d9 were prepared in a concentration of 8.8 mmol/L and 5.2 mmol/L by dissolving the compounds in methanol:water (50:50 v/v).

The protein precipitation solution consisted of MeOH/ACN 1:1 (v/v) to which the I.S. of choline-d9 and phosphocholine-d9 were

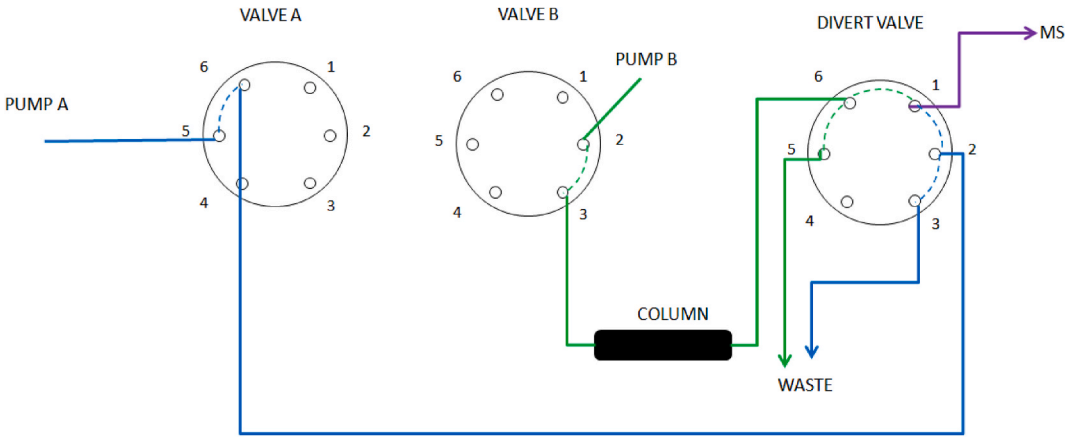

Fig. 1. Scheme of valve connection of HPLC-MS system. Valve A connects pump A to the Divert valve (blue line); Valve B drives the mobile phase managed by the pump B to the chromatographic column (green line); Divert valve guides the flux from pump A and B into the mass spectrometer (purple line) or to the waste.

added to obtain a concentration of 1.10 μmol/L and 0.13 μmol/L, respectively.

Calibration solutions were made from the stock solutions of analytes and I.S. The standard curve was obtained by serial dilution of a solution containing 38.40 μmol/L of choline and 5.43 μmol/L of phosphocholine.

Once prepared, all solutions were stored at −20 °C until analysis.

2.3. Equipment and HPLC–MS/MS conditions

The analysis was performed with HPLC-MS/MS technique in Selected Reaction Monitoring (SRM) mode, using the mass spectrometer triple quadrupole Quantiva from Thermo Scientific (Massachussetts, USA) coupled with LC Transcend Thermo Scientific (Massachussetts, USA), including an autosampler, two quaternary pumps (Fig. 1S) and column oven. The chromatographic separation was performed with the HILIC column Atlantis premiere BEH Z-HILIC 2.1 × 100 mm 2.5 μm FIT from Waters (Massachusetts, USA). The column temperature was set at 30 °C. The flow rate of the eluting pump (pump B) connected to the valve B was 0.45 mL/min with mobile phase A (NH_4HCO_3 10 mM pH 8.5) and phase B (Acetonitrile/Methanol 90:10 v/v). The gradient consisted of 20 % solvent A and 80 % solvent B for 0–4.02 min, followed by a step of 50 % solvent A and 50 % solvent B held to 10.68 min in isocratic mode. Then, initial conditions were established through a linear gradient from 10.68 to 11.68 min and then kept constant for 3 min to re-equilibrate the column. The washing pump (pump A) was connected to Valve A held a 100 % of phase C (ACN/MeOH/Acetone 60:30:10 v/v) with a flow rate of 0.4 mL/min in the range 10.68–13.68 min. The divert valve, initially in 1-2position, was switched in 1–6 position at 11.68 min (Table 1S and Fig. 1). At 13.68 min it was switched back to 1–2 position till the end of run (Fig. 2). The total run time of analysis was 14.68 min.

Mass detection was performed using a heated electrospray ionization (H-ESI) in positive mode under the following condition: HESI spray voltage 3.3 kV, sheat, auxiliary and sweep gases were set to 40, 15 and 0 arbitrary units respectively. Ion transfer tube temperature was set to 333 °C and vaporizer temperature to 317 °C. Selected Reaction Monitoring (SRM) were run for the analytes most abundant fragment 184 → 125 m/z transition for phosphocholine (collision energy 19.7 V), 193 → 125 m/z for phosphocholine-d9 (21.1 V), 104 → 60.1 m/z for choline (18.1 V), 113 → 69 m/z for choline-d9 (20.1 V) as shown in Table 1. Meanwhile, 184 → 86.2 m/z (collision energy 18.7 V) and 104 → 45.2 m/z (collision energy 21.96 V) were used for quality identification of PCho and Cho, respectively. Internal standard peak area was used for area normalization to monitor system performance throughout analysis. Data processing was conducted by using Excalibur software from Thermo Scientific (Milan, Italy).

2.4. Sample preparation

In-house pooled plasma was constituted using leftovers of anonymised plasma samples obtained from volunteers at overnight fasting who, in any case, expressed written consent for its use. Pool plasma samples were aliquoted and stored at −20 °C.

2.5. Analytical procedure

Plasma aliquots were thawed at room temperature. Protein precipitation was achieved by adding 1600 μL of cold precipitation solution containing I.S. to 400 μl of plasma sample and centrifuging at 3000 rpm at 4 °C for 15 min. The supernatant was transferred into a polypropylene tube and then dried with vacuum centrifuge. Finally, dried extracts were reconstituted with 100 μL of ACN/H_2O (1:1 v/v). ACN/H_2O (1:1 v/v) was also used as blank sample. Five μl of both plasma samples and blank samples were injected into the chromatographic system.

2.6. Validation procedure

The reliability and the acceptability of the analytical method was verified through a partial validation. According to European Medicine Agency (EMA) Guidelines [24], Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, accuracy, specificity, inter-day precision and intra-day precision were tested.

2.6.1. Linearity

Linearity was evaluated by standards compound dilution. Seven serial 1:2 dilution in water were prepared to obtain a series of

Table 1
Mass spectrometer parameters set for SRM detection mode.

Analyte	Parent ion (*m/z*)	Product ion (*m/z*)	Collision Energy (V)	RF Lens (V)
Choline *	104.1	45.2	21.96	47
Choline #	104.1	60.1	18.1	47
Choline – d9	113.1	69.2	20.1	48
Phosphocholine *	184.1	86.2	18.7	57
Phosphocholine #	184.1	125	19.7	57
Phosphocholine – d9	193.1	125	21.1	58

The resulting peak is used for quantification purposes. * The resulting peak is used for quality purpose.

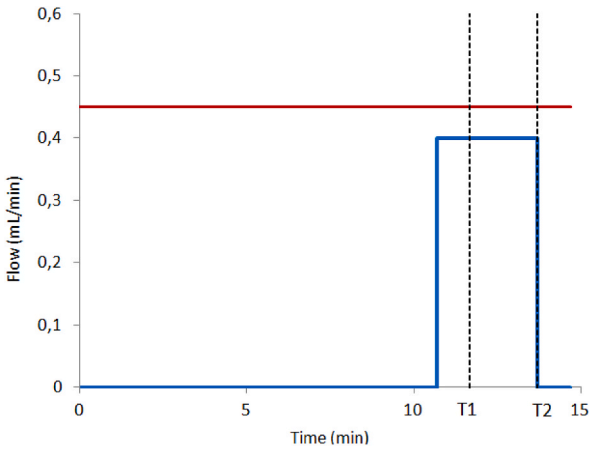

Fig. 2. Flow rate of pump A (blue line) and pump B (red line), where T1 and T2 represents the moment of the switch of the divert valve (from 1 to 2 to 1–6 position at 11.68 min, and from 1 to 6 to 1–2 at 13.68 min).

solutions with a concentration range from 5.43 μmol/L to 0.08 μmol/L of phosphocholine, and from 38.40 μmol/L to 0.60 μmol/L of choline. Each diluted standard was analyzed in three days, preparing different stock solution each day. Calibration curves were fitted by a linear model of the median standard concentration, and linearity was evaluated by the coefficient of determination R^2.

2.6.2. Limit of Detection and limit of quantification

LOD and LOQ were evaluated through the analysis of three blank samples performed on three consecutive days, for a total of 9 samples tested. Their values were calculated as follows:

$$LOD = BLK_{conc} * 3S \; LOQ = BLK_{conc} * 10S$$

where BLK_{conc} is the mean concentration of the nine (three for each analytical session) blank injections and S is the standard deviation of the blank concentration.

2.6.3. Accuracy and specificity

The accuracy and specificity of the method were evaluated through repeated measurements of Cho and PCho at different levels as specified in the EMA Guidelines. LLOQ (lower limit of quantification), low, medium and high concentrations were obtained by spiking plasma with 0, 1.92, 7.68, 23.04 μmol/L of Cho standard solution and 0, 0.27, 1.09, 3.26 μmol/L of standard Cho standard of PCho. Choline and phosphocholine standards were mixed into the samples after the addition of protein precipitation solvent and before centrifugation. Each level was tested in triplicate each day for three consecutive days. Method accuracy was calculated as the recovery percentage of the spiked Cho and PCho in plasma sample as prepared in paragraph 2.5. The recovery was calculated with the following formula

$$\%\text{Recovery} = C_x \,/\, (C_0 + C_{spk}) * 100$$

where C_x is the average cholines concentration detected in the spiked plasma analyzed in triplicate for three days, C_0 is the average concentration of the plasma sample (LLOQ) analyzed in triplicate for three days, and C_{spk} is the spiked concentration. These values were also used to evaluate selectivity.

Furthermore, the discrimination of Cho and PCho from interferents that are present in the matrix will be evaluated by examinating the chromatograms. Peaks in sample chromatogram whose retention times differ from the ones detected in standard chromatogram have been inspected.

2.6.4. Precision

Method precision was evaluated in terms of coefficient of variation percentage for both intra-day and inter-day of estimated concentration at high, medium, low, and LLOQ.

For both Cho and PCho, a variance components analysis was applied to estimate total peak areas variability associated with the day of sampling (inter-day) and the replication over each sample (intra-day). Coefficients of Variation (CV) were determined for the intra-day and inter-day variability by means of the PROC GLM procedure of SAS®, Version 9.4 [25].

3. Results

Fig. 3a shows a typical LC-MS chromatogram of Cho and PCho standards dissolved in aqueous matrices. The peaks detection was obtained monitoring the fragmentation of 184 → 125 and 104 → 60 in SRM mode. The retention times (RT) were 5.6 min for PCho (red

line) and 5.74 min for Cho (black line). Similarly, Fig. 3b shows a chromatogram obtained by the analysis of a pooled human plasma sample, in which the fragment previously described are monitored.

3.1. Method validation

3.1.1. Linearity

The calibration curve range was defined according to choline and phosphocholine concentration in human plasma as reported by Guasch-Ferré and co-workers [26]. The linear model showed an average R^2 coefficient of 0.998 for Cho and 0.999 for PCho, showing excellent linearity within the physiological range of the analytes (Fig. 4).

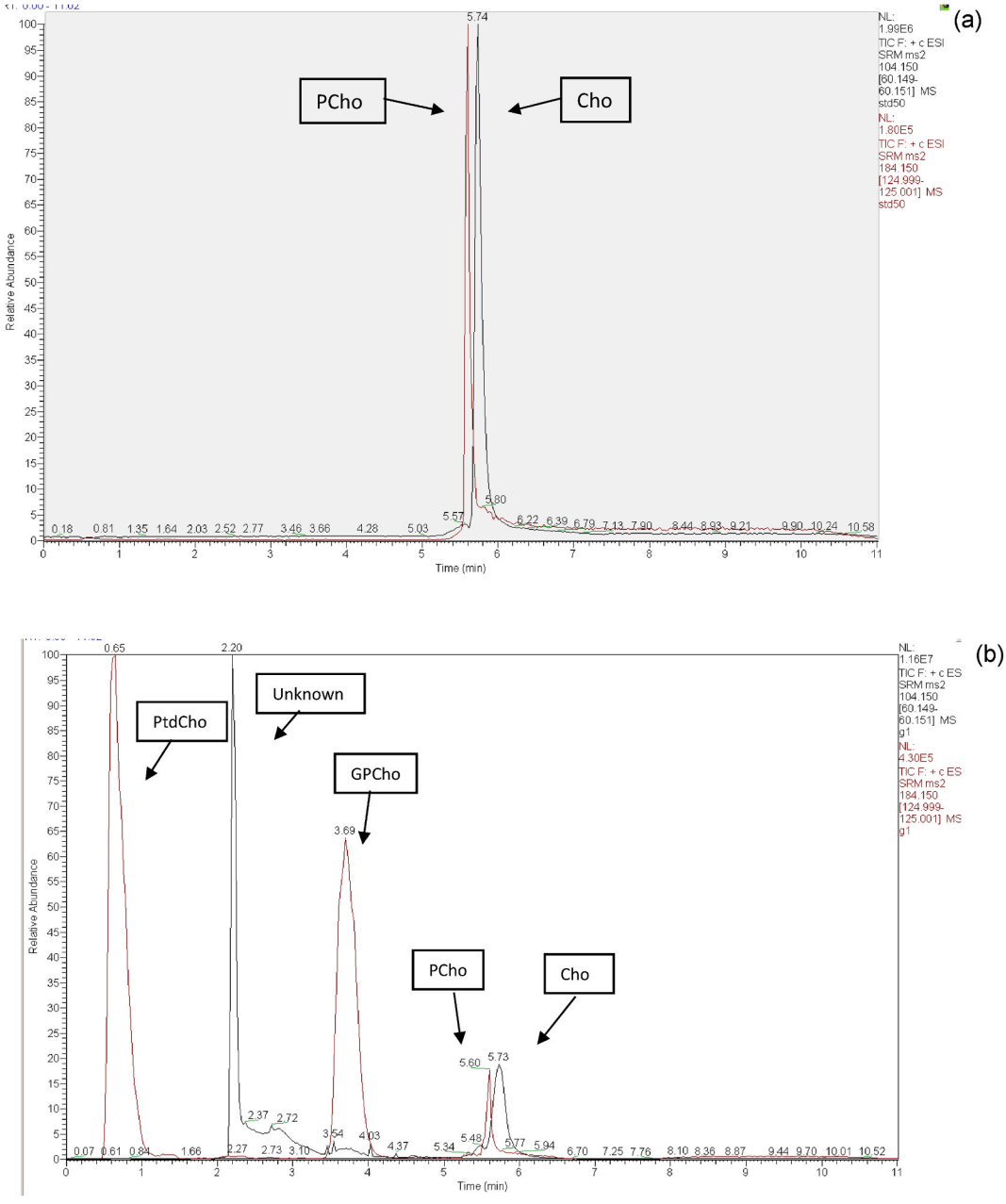

Fig. 3. (a) Cho and PCho aqueous standard 0.27 μmol/L chromatogram. The analytes are detected trough the monitoring by SRM mode of 104 (black line) and 184 (red line) *m/z* respectively; (b) 104 (black line) and 184 (red line) *m/z* SRM chromatogram in a pooled human plasma sample.

3.1.2. Limit of Detection and limit of quantification

The established instrumental setting allows to achieve a LOD of 0.06 μmol/L and 0.04 μmol/L for Cho and PCho respectively, and a LOQ of 0.19 μmol/L and 0.11 μmol/L for Cho and PCho respectively. These parameters fully satisfied the requirements of detectability of the physiological concentrations of the two cholines.

3.1.3. Accuracy and specificity

The results of accuracy evaluated in terms of recovery percentage of spiked Cho and PCho are shown in Table 2. The spiked choline recovery percentage ranged from 97.0 % to 99.8 % for Cho and 84.7 % and 96.0 % for PCho, meeting the requirements of the EMA Guidelines both for accuracy and specificity [24]. Specificity was evaluated through the examination of the sample chromatogram. As shown in Fig. 3, the retention time of Cho and PCho in plasma corresponds to that of aqueous matrix standards. Moreover, in our plasma pool we observed the presence of other peaks originated by the same SRM of cholines, namely, RT 0.65, 2.2 and 3.69 min (Fig. 3b). These peaks were identified as phosphatidylcholine (RT 0.65 min), glycerophosphocholine (RT 3.69 min) (Fig. 1S), while the peak at 2.2 min was not identified.

3.1.4. Precision

Results of variance components analysis are shown in Table 2. Intra-day CV is stable for all the concentration levels. Nevertheless, it is lower than the 15 % benchmark. Intra-day CV ranged between 2.2 % and 4.1 % for Cho and between of 3.2 % and 15 % for PCho.

As concerns inter-day precision, the CV lies below the 15 % benchmark ranged from a minimum value of <1 % and 6.5 % for Cho. CV for PCho is 20 %.

3.1.5. Concentration values in human plasma

The plasma tested in this analysis was a pool of ten volunteers. The average circulating value was 15.97 μmol/L for choline and 0.34 μmol/L for phosphocholine (Table 2). These values were obtained from the aliquots of non-spiked plasma pools.

4. Discussion

This paper described a fast and robust analytical method to quantify choline and phosphocholine in human plasma by LC-MS/MS.

Although the described method presents an analytical pipeline similar to others in the literature [26,27], it reports interesting innovative aspects. The first is related to the performance of the new generation HILIC_zwitterionic column capable of returning excellent analytical resolution of the two Chos under study. The other aspect concerns the strategy adopted to intensify the cleaning of the column, thus allowing the quality of the chromatographic peak to be increased and the sample carry-over effects (,i.e. the residual sample left over from a previous injection) to be reduced.

4.1. Instrumentation setting

4.1.1. HPLC system

Before using a zwitterionic HILIC column, the C18 column Atlantis C18 AX 2.5 μm 2.1 × 100mm MPFIT (Waters, Massachusetts USA) was tested. By using a gradient mobile phase composed of ammonium bicarbonate buffer at pH 7 (phase A) and acetronitrile (phase B) Cho and PCho eluted within 1 min (data not shown). Starting the gradient with a higher percentage of water did not improve the resolution. Increasing acetonitrile in mobile phase broadened the peaks without improving the peak separation. On the contrary, the Atlantis premiere BEH Z-HILIC column performed well thanks to its zwitterionic nature which allows polar analytes to strongly interact with the stationary phase. Mobile phase composition, pH and ionic force were carefully tuned. In HILIC columns, aqueous and organic solvents must be miscible in order to guarantee that the partition equilibrium is established. Analytes which bear pH dependent moieties are strongly influenced by mobile phase pH [21]. Cho and PCho had a pH-independent positive charged quaternary amine, and a hydroxyl and phosphate group respectively, which are pH dependent. Cho has a pka = 13.97 [28] and PCho has pka = 1.15 [28]. Several pH solutions were tested within pH range which allowed the ion form of the analytes to be kept unchanged

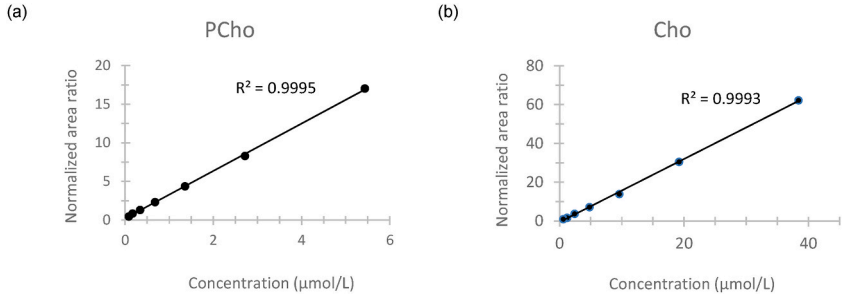

Fig. 4. (a) PCho calibration curves expressed in μmol/L vs Normalized area ratio; (b) Cho calibration curves expressed in μmol/L vs Normalized area ratio.

Table 2
Choline and phosphocholine assay precision, accuracy, LOD and LOQ measured in a pool of human plasma.

	Spiked standard (μmol/L)	Mean concentration# (μmol/L)	Intra-day CV (%)	Inter-day CV (%)	Accuracy (%)	LOD (μmol/L)	LOQ (μmol/L)
Cho	0	15.97	4.1	6.5	–	0.06	0.19
	1.92	17.36	4.1	<1	99.8		
	7.68	23.30	2.2	3.5	98.5		
	23.04	38.94	2.7	2.8	97.0		
PCho	0	0.34	3.2	8.5	–	0.04	0.11
	0.27	0.52	10.3	20.0	84.7		
	1.09	1.23	15.0	12.9	86.5		
	3.26	3.46	4.4	6.2	96.0		

Three-days triplicate average concentration of Cho and PCho in human plasma sample.

and according to the manifacturer's the column suggested pH range. The tested mobile phase and pH were ammonium formate buffer 20 mM pH 3.8 which is often described in literature [29,30], ammonium acetate buffer pH 6.5, and ammoniumhydroxybicarbonate 10 mM pH 7.6 and 8.5 (Fig. S2). Although the use of low pH mobile phase for the separation of Cho and PCho is reported in several articles [29–31], in our experience PCho peak tailing issues emerged by using acidic mobile phase. This may be due to the phosphate group interacting with the metallic components of the instrument [32,33]. In particular, phosphate groups can be absorbed on stainless steel surfaces of the injection system, the column and the mass spectrometer ESI probe, especially when surfaces are scratched or corroded. Tailing was limited by run multiple needle washes, as described in paragraph 2.3. It has been reported that phosphoric acid, phosphate buffer or etheylenediaminetetraacetic acid (EDTA) may suppress this interaction. However their use in mass spectrometry may provoke loss of sensitivity, ion source contamination, ion suppression, and ESI probe clogging due to salts formation [32,34]. Using basic mobile phase can be beneficial in preventing this interaction without a negative impact on the mass spectrometer performance. In our experience, PCho peak shape improved by using a 8.5 pH buffer.

4.2. Validation procedure

To our knowledge, this is the first study which providing analytical validation of the simultaneous detection of choline and phosphocholine in human plasma by HPLC-MS. The HPLC-MS based assay exhibited good accuracy and precision, in accordance with EMA Guidelines [24].

Initially, phosphocholine carryover effect affected the tests to determine the LOD and LOQ values in terms of high signal noise ratio in the blank samples (data not shown). We hypothesize that the phosphate group interacts with metal component on HPLC-MS systems, as previously reported [32]. In this method cleaning steps of the column and of the ESI probe are run simultaneously with different solutions. Unlike common HPLC-MS systems provided by a single pump, the system described allows us to use two separate quaternary pumps (Fig. 1). In this way, pump A is able to dispense aggressive solvent mixtures appropriate for the ESI probe cleaning avoiding column degradation or long re-equilibration times.

Specificity of the method is tested by inspecting the Selected Reaction Monitoring (SRM) chromatogram for Cho and PCho. In addition to the peak detected in the standard chromatogram, in plasma samples other peaks are detected as reported in section 3.1.3 It is known that various endogenous molecules, such as the abundant class of phospholipids, could fragment at the HESI source level, losing their headgroups [35,36], and giving rise to fragments that could correspond to Cho and PCho ones. These fragments may lead to multiple peaks at 104 and 184 m/z, which can hardly be distinguished from those originated by choline and phosphorcholine standards. Nevertheless, different retention times may separate and resolve the selected analyte from interferents.

More specifically the peak eluted at 0.65 min is probably due to phosphatidylcholine in-source fragmentation, whose major fragment is the phosphocholine ion with m/z 184 [37]. This hypothesis is confirmed by the early elution on the top of the column. In fact, phosphatidylcholines are not well retained by HILIC column due to their high hydrophobicity. Furthermore, the abundant peak observed is consistent with the high concentrations of these substances in the blood, as described by Quell and co-workers [38]. The peak detected at 3.69 min both in Cho and PCho (Fig. 1S a) is identified as Glycerophosphocholine (GPC) as the most intense peak registered in full scan mode is 258 m/z and its fragmentation pattern (60, 86, 99, 104, 124, 166 and 184 m/z, collision energy 30 V) (Fig. 1S b) coincides to the GPC fragmentation pattern available in Human Metabolome Database [39].

GPC exhibits a mild in-source fragmentation as 104 and 184 peaks are detected at 3.69 min. There is no identification hypothesis for the peak detected at 2.20 min.

In repeatability and recovery tests, different standard quantities of Cho and PCho are added to plasma samples. PCho recovery is strongly dependent from the procedure adopted, as reported in Table 2S and Fig. 3S. Interestingly, the standard spiking step is crucial. In particular, recovery improves when the protein precipitation solvent is added before the standard phosphocholine spiking. We hypothesize that in plasma an appreciable enzymatic activity capable of degrading PCho persists. Further studies regarding phosphocoline interactions with plasma metabolites are needed. Thus, in order to avoid inaccurate determination, sample treatment must be performed following strict standardized procedures which must take into account the sampling and sample handling steps.

The levels of Cho and Pcho detected in our unspiked plasma pools of apparently healthy volunteers, (15.97 μmol/L for choline and 0.34 μmol/L for phosphocholine), are substantially aligned with those in the literature. Guasch-Ferré et al. [28], using a LC/MS method for the simultaneous measurement of the 2 cholines in 751 subjects, found mean circulating values of 23.17 μmol/L for Cho and 0.59

μmol/L for PCho.

While the measurement of PCho in human plasma by HPLC-MS are scarce [26], there are several works that report the values of circulating Cho concentrations in healthy subjects, measured with different techniques. For example, Nitter et al. [13] analyzing choline concentration in citrate plasma of 2323 healthy subjects by LC/MS method, found Cho ranged from 6.2 to 14.2 μmol/L. This range was consistent with that found by Garcia et al. [20], measuring Cho in 541 EDTA plasma sample with the NMR technique (9.0–13.1 μmol/). Otherwise, Cho values measured with a 60 plasma-heparin samples chemiluminescent assay fell within a wider range (1.4–34.4 μmol/L) than the aforementioned methods.

5. Conclusion

In this paper a fast and robust target analytical method was validated to simultaneously quantify choline and phosphocholine in human plasma by LC-MS/MS.

The use of innovative HPLC-columns such as HILIC, particularly suitable for small and highly polar molecules, has made possible an effective separation and consequently the quantification of the two cholines. An additional analytical step that contributes to the accuracy of the method, consists in intensive cleaning of the mass spectrometer which increases peak quality and reduces carryover effects. These technical strategies allowed us to obtain a fast and effective method particularly suitable for the study of the two cholines in the clinical setting.

Declarations

All participants/patients (or their proxies/legal guardians) provided informed consent to participate in the study.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Giulia Guerra: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Francesco Segrado:** Writing – review & editing, Validation, Resources. **Patrizia Pasanisi:** Writing – review & editing. **Eleonora Bruno:** Writing – review & editing. **Salvatore Lopez:** Conceptualization. **Francesco Raspagliesi:** Conceptualization. **Michela Bianchi:** Validation. **Elisabetta Venturelli:** Writing – review & editing, Visualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21921.

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