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Ion-exchange liquid chromatography method with indirect UV detection for the assay of choline in pharmaceutical preparations*

PIERRE LEROY,‡ MURIELLE BARBARAS,‡ JEAN LUC COLIN§ and ALAIN NICOLAS†‡

‡*Laboratoire de Chimie Analytique, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques — B.P. 403, 54001 NANCY Cedex, France*
§*Centre de Recherche Vétoquinol S.A., MAGNY-VERNOIS — B.P. 189, 70204 LURE Cedex, France*

Abstract: A high-performance liquid chromatography (HPLC) method coupled with indirect UV spectrophotometric detection was developed for the routine control of pharmaceutical preparations containing choline. Optimization of the HPLC system using silica-based cation exchange columns was focused on the combination in the mobile phase between a UV transparent co-ion and a UV active cationic probe. The selected mobile phase consisted of a mixture of ethanol-ammonium acetate buffer (100 mM, pH 5.0) (20:80, v/v) containing 0.3 mM of 3-hydroxytyramine as chromophoric probe; UV detection was operated at 280 nm. The HPLC system provided full selectivity with regard to the multi-ingredient pharmaceutical matrices tested. Quantitative analysis was validated by testing linearity of the method between 50 and 150% of the theoretical content (coefficient of correlation greater than 0.99) and repeatability (relative standard deviation less than 2.5%; $n = 5$).

Keywords: *Choline; pharmaceutical preparation; ion-exchange chromatography; indirect photometric detection; UV absorbing co-ion.*

Introduction

Choline (trimethylhydroxyethylammonium) is used in pharmaceutical preparations (i.e. parenteral and oral solutions, syrups), especially those active against anaemies, hypovitaminic states and haepatic diseases. Methods usually available for routine control include colorimetric determination with ammonium reineckate or gravimetric determination with tetraphenylborate, phosphotungstic acid or aluminium chloride [1]. However, these methods lack selectivity for testing complex matrices. So separative techniques have to be applied. Major problems encountered to develop GC and HPLC techniques are respectively due to the low volatility of choline and the absence of a chromophore in its structure. Since the original report describing the determination of acetylcholine and choline using HPLC with post-column enzymatic reactions and electrochemical detection of produced hydrogen peroxide [2], multiple subsequent papers have clearly demonstrated the usefulness of this approach in biological matrices. However, this technique is too hyphenated for monitoring the quality control of pharmaceutical preparations and so other approaches for the assay of choline using HPLC have to be considered. A pre-column derivatization of choline followed with an ion-pairing reversed phase separation of the resulting 3,5-dinitrobenzoate ester was applied to its measurement in plasma [3]. More recently, an ion-pairing technique in reversed phase elution mode using a UV absorbing co-ion, i.e. *p*-aminobenzenesulphonic acid, and indirect spectrophotometric detection has been reported [4].

This paper describes the development and optimization of an ion-exchange HPLC technique coupled with indirect UV detection and devoted to the measurement of choline in pharmaceutical preparations.

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†Author to whom correspondence should be addressed.

Experimental

Chemicals and reagents

Choline chloride (M_r = 139.63) was obtained from Aldrich (Steinheim, Germany) and met the specifications of the European Pharmacopoeia. UV absorbing compounds, i.e. 9,9′-bis-N-methylacridinium nitrate, 3-hydroxytyramine hydrochloride were purchased from Aldrich and promethazine hydrochloride from Cooper (Melun, France). The two pharmaceutical dosage forms tested were commercially available solutions for veterinary use and were obtained from Vetoquinol Laboratories SA (Magny Vernois, France). Their respective content in choline was 50.0 g l^{-1} (formulation 1) and 18.0 g l^{-1} (formulation 2). Aminoacids, water- and fat-soluble vitamins and polyols were the other associated active substances. Parabens were used as preservatives.

All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Apparatus

The HPLC system consisted of a two solvent gradient pump (model P 2000, Thermo Separation Products, Les Ulis, France), an autosampler including an injection valve equipped with a 20-μl loop (model SP 8775, Thermo Separation Products), a column oven (model Croco-Cil Thermo Separation Products); detection was operated with either a UV-visible variable wavelength spectrophotometric detector (model UV 2000, Thermo Separation Products) or a refractometric detector (model 410, Millipore-Waters, Saint-Quentin-en-Yvelines, France). All data collection and calculations were performed using an integrator (model Chromjet, Thermo Separation Products) connected with a software (model "Winner on Windows", Thermo Separation Products).

UV spectra and absorbance of probes were measured using 1-cm cuvettes with a UV-visible variable wavelength spectrophotometer (model DU 64, Beckman, San Ramon, CA, USA).

Chromatographic conditions

The prepacked cation-exchange columns (Zorbax SCX 300 Å, 5 μm, 300 × 4.6 mm i.d.; Life Sciences International, Cergy Pontoise, France and Nucleosil SA 100 Å, 5 μm, 125 × 4 mm i.d.; Macherey Nagel, Düren, Germany) were eluted with mixtures of ethanol and various buffers (20:80, v/v) at a flow rate of 1.2 ml min^{-1} and at a column temperature of 40°C. Buffers were prepared from potassium hydrogenophosphate, ammonium acetate and ethylene diamine hydrochloride solutions at different concentrations; the pH value was adjusted at 5.0 using the corresponding concentrated acid solution. Detection was operated either with refractometry (no probe was added in the mobile phase) or with UV spectrophotometry at the maximum absorbance wavelength according to the probe incorporated in the mobile phase.

Current polarity between the detector and the integrator was reversed to enable choline peak to be displayed in the positive direction and to make integration of peak areas easier.

For validation procedure, the mobile phase was ethanol–ammonium acetate (100 mM, pH 5.0) (20:80, v/v) added with 0.3 mM of 3-hydroxytyramine hydrochloride and UV detection was operated at 280 nm.

After performing analysis, the column was washed with ethanol–water (20:80, v/v) and kept under ethanol–water (70:30, v/v) when not used.

Quantitative analysis

Stock solutions of choline chloride were prepared in HPLC grade water at respective concentrations of 12.5 and 18.0 g l^{-1} according to the formulation tested; further dilutions were made in the mobile phase. Calibration curves were calculated using a placebo of the pharmaceutical preparations (containing all ingredients but choline) fortified with choline levels of 50.0, 75.0, 100.0, 125.0 and 150.0% of the theoretical concentration and further diluted in the mobile phase. Sample dilutions of the formulation 2 containing fat-soluble vitamins were passed through a Sep Pak C_{18} cartridge (Millipore–Waters) previously activated with 2 ml of methanol and 2 ml of HPLC grade water, before injection onto the HPLC system. Final concentration of injected solutions ranged from 1.25 to 3.75 (formulation 1) and 0.9 to 2.7 g l^{-1} (formulation 2), respectively.

Results and Discussion

Many HPLC methods have been reported for the determination of UV transparent

analytes with indirect UV detection. Most of them have been developed using reversed phase columns and mobile phases including a chromophoric counter-ion with an ion-pairing mechanism [5, 6].

When using ion-exchange liquid chromatography coupled with UV indirect detection, the role of the UV active co-ion is also in the deplacement of a UV transparent ionic analyte as a dip or a trough in the baseline, as first demonstrated by Laurent and Bourdon [7] then more extensively developed [8, 9]. When the only co-ion present in the mobile phase is the chromophoric probe, the ionic analyte displaced an equivalent amount of this co-ion, so that the electroneutrality of the mobile phase is maintained. Consequently, the eluting transparent analyte ion will then appear as a disturbance of the baseline. This technique has been applied to anionic [10, 11] and cationic [12–14] species.

Two different approaches for ion-exchange chromatography coupled with UV indirect detection of transparent cationic species have already been reported in the literature: (i), a single co-ion with chromophoric properties is used in the mobile phase for elution and detection [12, 13], (ii), a UV absorbing co-ion is added to the mobile phase containing another UV transparent co-ion which permitted the elution of cationic substances [14]. In this case, competition occurred between the anionic sites of the stationary phase and the positively charged analyte and two co-ions.

The later approach has been used in the present study devoted to choline, a quaternary ammonium compound. The development has been made according to the following steps:

(i) optimization of eluting conditions of choline using refractometry as detection mode;

(ii) choice of a UV absorbing co-ion with suitable spectral and chromatographic properties;

(iii) selection of optimized chromatography and detection conditions and validation of the method.

A detailed scheme of the overall HPLC assay development is shown in Fig. 1.

Optimization of eluting conditions of choline

Silica-based stationary phases bonded with sulphonic acic groups as cationic exchangers have been used in the present work. All the experiments during the development were performed using Zorbax SCX columns. The optimization has been limited to changes in the mobile phase composition, but pH of the buffer solution (5.0) and ethanol content (20%, v/v) have been kept at constant values in the different mobile phases tested. The most suitable conditions for choline elution have been determined by evaluating the influence of the concentration of different co-ions, i.e. potassium, ammonium and ethylenediamine. Refractometry was used for the detection of choline during this development step. High concentration of potassium was needed to elute choline, so this co-ion was rejected. A linear relationship between $1/k'$ (capacity factor) and ammonium and ethylenediamine concentrations in the mobile phase was established (Fig. 2), which demonstrated an ion-exchange process for choline elution. Otherwise, ammonium and ethylenediamine afforded convenient k' values (ranging from 1 to 3) and tailing factors (less than 1.5) for choline peak, with ionic strength (10 to 100 mM) of corresponding mobile phases resulting in acceptable back-pressure on the column (<1500 p.s.i.). However, numbers of theoretical plates were higher with ammonium (ca 30 000 m^{-1}) than with ethylenediamine (<20 000) and so ammonium was chosen.

Choice of the UV absorbing co-ion

The high-capacity cationic exchanger used in the present study implies the inability of selecting a UV absorbing co-ion at an appropriate concentration which could displace choline from the stationary phase without saturating the signal of the spectrophotometric detector. So, a UV co-ion was added to the mobile phase containing a high concentration of ammonium ions.

Different UV absorbing probes, i.e. 3-hydroxytyramine, promethazine and 9,9′-bis-N-methylacridinium, were evaluated to obtain a sufficient sensitivity for the measurement of choline in the HPLC system coupled with indirect spectrophotometric detection. Their ionization constant, their spectrophotometric properties and their k' values obtained in the present ion-exchange system are indicated in Table 1. They are present as cationic species in the mobile phase conditions and so are retained on the cationic-exchanger column.

The sensitivity of the response obtained for choline with UV indirect detection was measured for each probe (Fig. 3). The concentration of the probe added to the mobile phase

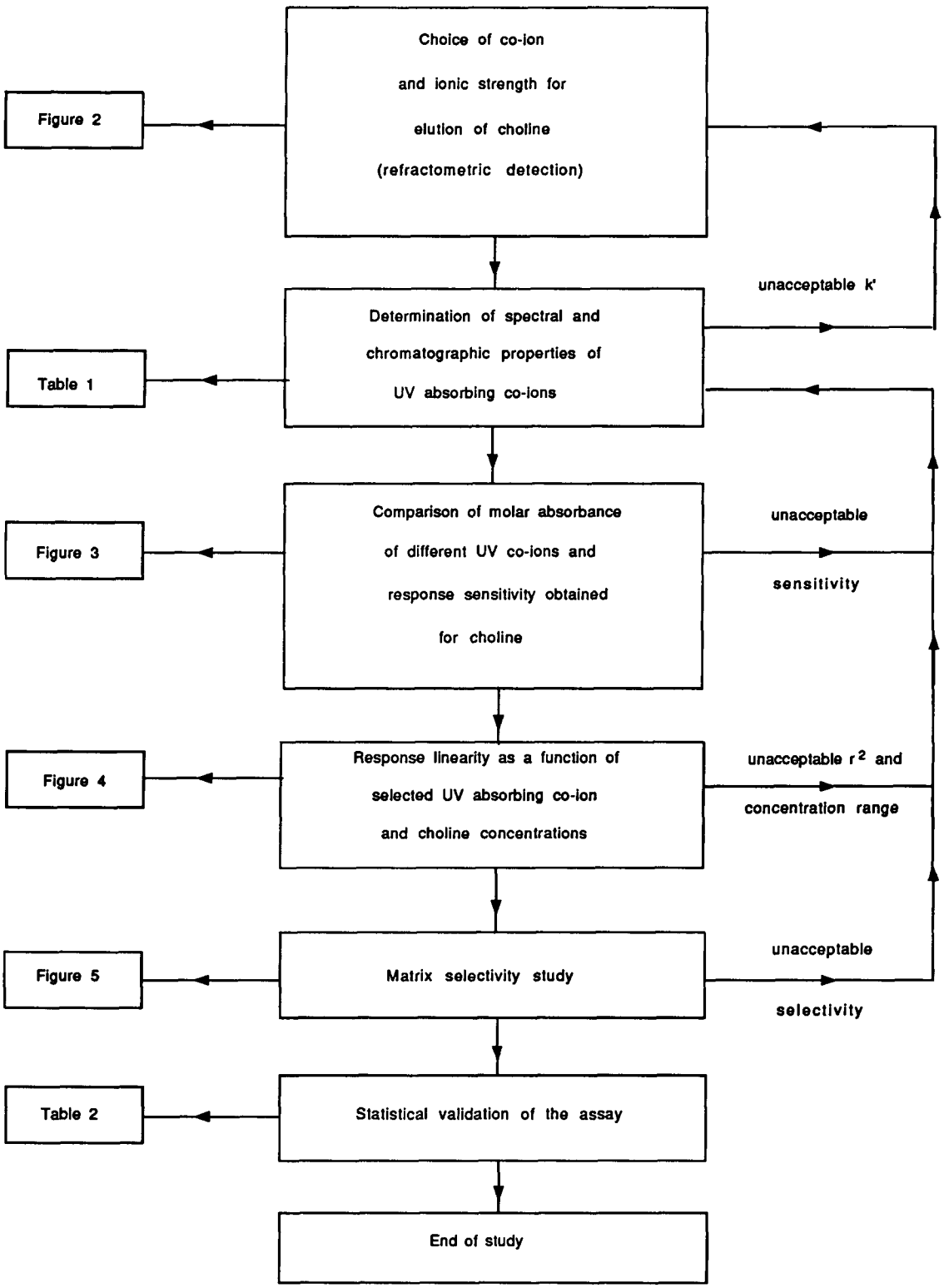

Figure 1
Scheme of the development of the HPLC system coupled with UV indirect detection for choline assay.

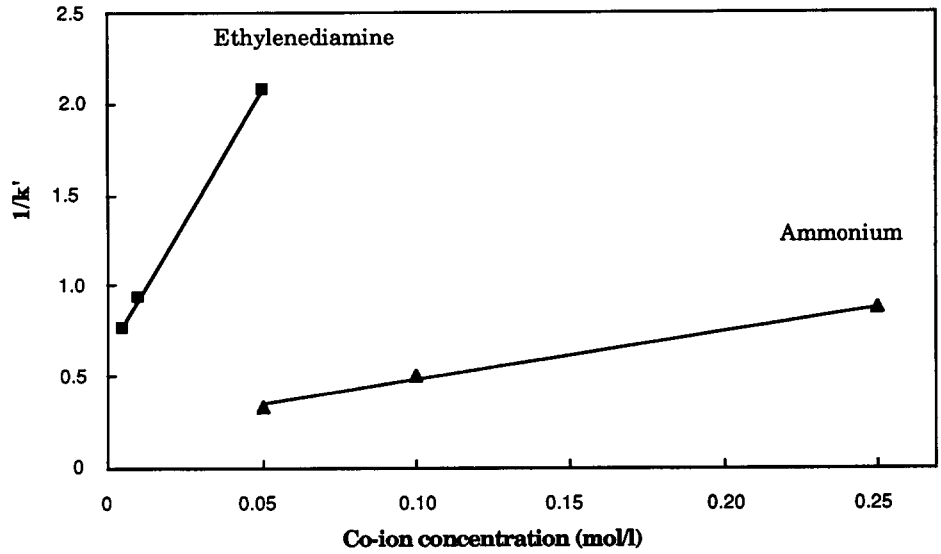

Figure 2
Variations of the capacity factor of choline as a function of the nature and the concentration of the eluting co-ion. ▲: ammonium; ■: ethylenediamine. (HPLC conditions: Zorbax SCX 300 × 4.6 mm i.d. column; mobile phase: ethanol–buffer pH 5.0 (20:80; v/v); flow rate: 1.2 ml min^{-1}; temperature: 40°C; detection: refractometry.)

Table 1
Ionization constants, spectral and chromatographic properties of choline and the different amino compounds tested as potential UV probes for indirect detection. (Chromatographic conditions as specified in Fig. 4)

	Ionization constant (pK_a)	λ_{max} (nm)	Molar absorbance (M^{-1} cm^{-1})	Capacity factor (k')
Choline	—	—	—	2.1
3-Hydroxytyramine	8.8	280	209	1.2
Promethazine	9.1	247	25,020	16.1
9,9'-bis-N-methylacridinium	—	350	1,430	>20

was calculated to obtain an absorbance close to 1 unit. Sensitivity (corresponding to the slope of the equation of the regression line between peak areas and choline concentrations) was not related to the molar absorbance of the probe. This fact may be explained as follows: a small and varying proportion of the ion-exchange sites, mostly occupied by ammonium ions, was occupied by the cationic probe. Moreover, it was observed that addition of any of the probes to the mobile phase did not modify the retention time of choline, which demonstrated that ammonium ions play the major role in the analyte elution. Promethazine was no more selected because it provided a too low sensitivity and the final choice of 3-hydroxytyramine was justified by a resulting lower baseline noise than with 9,9'-bis-N-methylacridinium.

Detection response for choline was tested as a function of the concentration of 3-hydroxytyramine (Fig. 4). The increase of its concentration gave higher sensitivity but the linearity range was shortened. The best compromise between these two factors was obtained for a 3-hydroxytyramine concentration of 0.3 mM. Eventually, the selected mobile phase consisted of ammonium acetate buffer (100 mM, pH 5.0) added with 20% (v/v) ethanol and 0.3 mM 3-hydroxytyramine.

Validation of the method

The experimental design consisted in placebo solutions fortified with choline at levels of 50.0, 75.0, 100.0, 125.0 and 150.0% of the theoretical content of the pharmaceutical preparations and assayed in five replicate runs according to the optimized procedure. Linearity, precision and accuracy have been evaluated (results in Table 2). The equations of the regression line between added and found contents were respectively: $y = 0.9988\ x -$

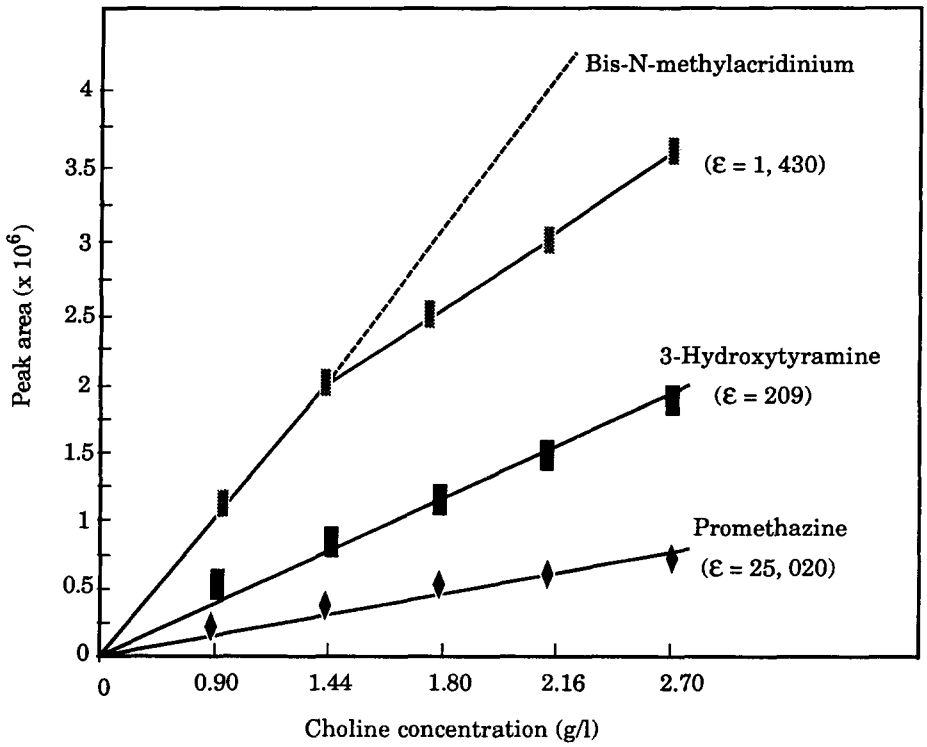

Figure 3
Sensitivity of the UV-indirect response (slope of the linear response measured at the maximum wavelength of the tested probe) as a function of the chromophoric co-ion added to the mobile phase. (HPLC conditions: Zorbax SCX 300 × 4.6 mm i.d. column; mobile phase: ethanol–ammonium acetate buffer (100 mM, pH 5.0) (20:80; v/v); flow rate: 1.2 ml min^{-1}; temperature: 40°C; detection: UV.)

Table 2
Statistical parameters obtained for the validation of the HPLC assay of choline in the pharmaceutical preparations

	Formulation 1					Formulation 2				
Added (% of label claim)	50.00	75.00	100.00	125.00	150.00	50.00	75.00	100.00	125.00	150.00
Found (% of label claim)	52.55	71.30	99.00	125.90	149.26	49.53	75.17	101.47	123.42	150.41
Recovery (%)	105.1	95.07	99.00	100.72	99.51	99.06	100.23	101.47	98.74	100.27
Relative standard deviation (%) $n = 5$	0.90	1.80	2.34	1.50	0.57	0.69	1.12	0.84	2.06	1.40

0.0860 ($r^2 = 0.9932$) and $y = 0.9999\ x - 0.0024$ ($r^2 = 0.9975$). ANOVA (Analysis of variance) demonstrated that slope and intercept values were not significantly different from 1.0 and 0.0. Specificity was demonstrated by injecting the placebo corresponding to each preparation: no interference with the choline peak was noted. Typical chromatograms are shown in Fig. 5. Moreover, the described HPLC system provided no late eluting system peak which have been previously mentioned for other ion-exchange techniques with indirect UV detection [15]. Consequently, it resulted in a high frequency of samples analysed (6–7 h^{-1}).

In order to test the ruggedness of the method, another silica-based cation-exchanger column, i.e. Nucleosil SA, was used and eluted with the same mobile phase: the resulting k' of choline was 4.6 and the sensitivity was the same. At least 100 samples could be analysed without noting a decrease of performances. Adsorption of hydrophobic compounds and matrix components was considerably reduced by a simple preparation step which consisted in a liquid/solid extraction using a C_{18} cartridge.

Conclusion

The present method appears to be valuable,

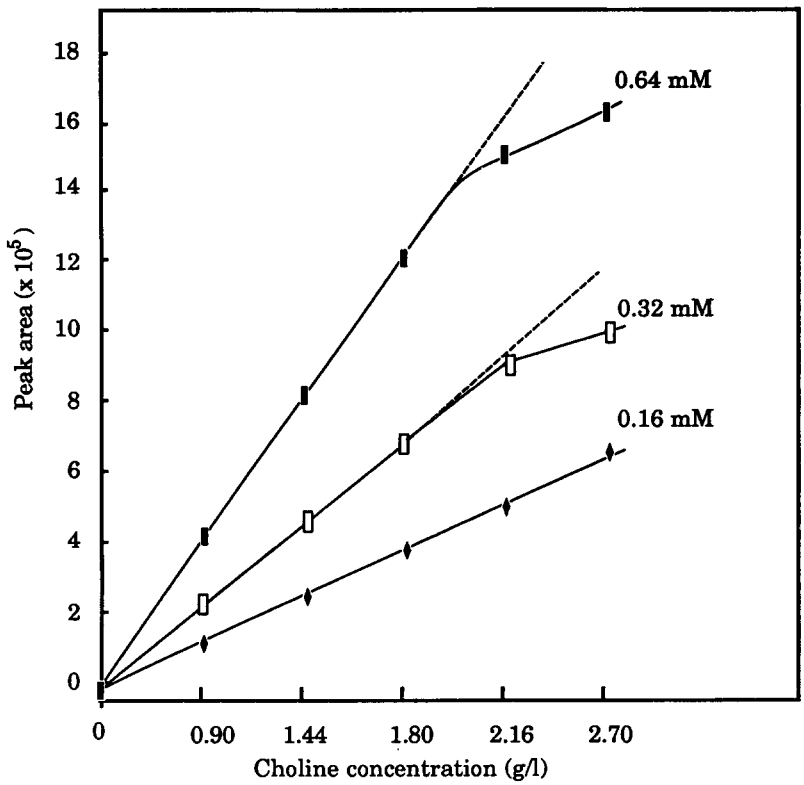

Figure 4
Linearity of the UV-indirect response as a function of 3-hydroxytyramine and choline concentrations. (HPLC conditions: Zorbax SCX 300 × 4.6 mm i.d. column; mobile phase; ethanol–ammonium acetate buffer (100 mM, pH 5.0) (20:80; v/v) added with 3-hydroxytyramine; flow rate: 1.2 ml min^{-1}; temperature: 40°C; detection: UV at 280 nm.)

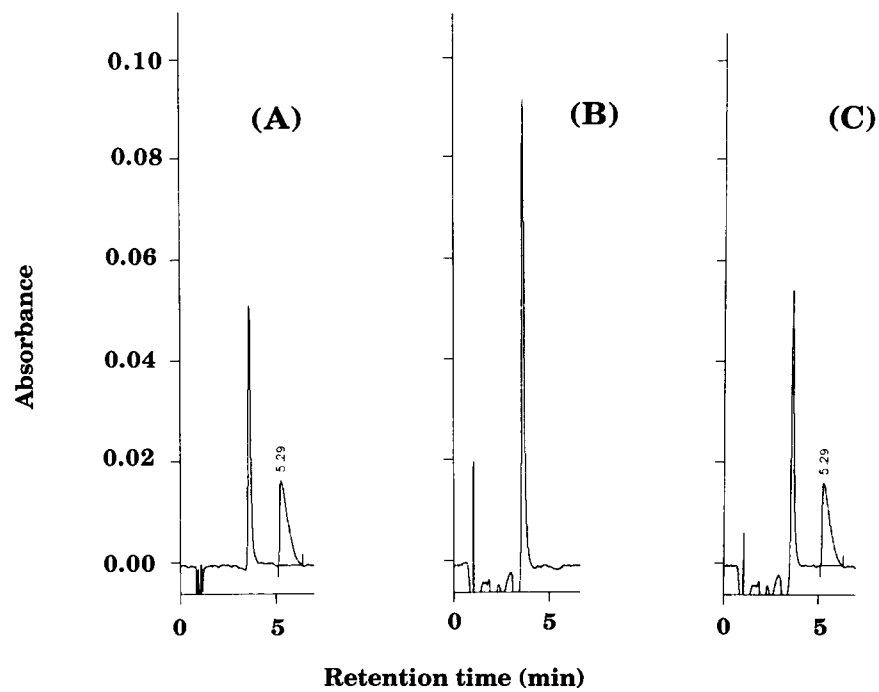

Figure 5
Typical chromatograms obtained wiht a standard solution of choline (1.8 g l^{-1}) (A), a placebo (B) and a placebo fortified with choline (C). (HPLC conditions: Nucleosil SA 125 × 4 mm i.d. column; mobile phase: ethanol–ammonium acetate buffer (100 mM, pH 5.0) (20:80; v/v) added with 0.3 mM 3-hydroxytyramine; flow rate: 1.2 ml min^{-1}; temperature: 40°C; detection: UV at 280 nm.)

giving fast (it avoids pre- or post-column derivatization), selective, reliable and accurate data. It provides a useful tool for the routine control of pharmaceutical preparations even though they contain many other UV absorbing ingredients. Moreover, the development scheme of the reported HPLC assay could be applied to other UV transparent ionic analytes.

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