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Chemiluminometric determination of choline-related substances in pharmaceutical preparations by dot-blot

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Abstract: A simple and reliable method of assaying succinylcholine chloride, oxtriphylline (choline theophyllinate) and acetylcholine chloride in pharmaceutical preparations, based on conversion to choline, is provided by combination of chemiluminometry of choline and dot-blot technique. Recoveries of 102 and 97% for choline chloride in Quelicin injection and Choledyl 200 tablets matrices, respectively, and of 99% for acetylcholine chloride in Miochol solution matrix could be achieved using simple choline chloride standards in phosphate buffer pH 8.6. Accordingly, matrix-matched standards were found redundant. Favourable results obtained in preparations-matched media including limits of detection of 37–39 pmol μl^{-1} of choline chloride, together with accuracies of 0–2% and RSDs ranging from 4 to 7% are the further evidence of the suitability of the method.

Keywords: *Succinylcholine chloride; oxtriphylline (choline theophyllinate); acetylcholine chloride determination; pharmaceutical preparations; choline chemiluminometry-dot-blot; direct standardization.*

Introduction

Numerous chemical assay methods have been developed to measure acetylcholine (Ach), choline (Ch), or both, in the same solution. Close to 30 different approaches have been reported, each focusing on some specific structural characteristic or biochemical feature associated with the Ach and/or Ch molecule. The currently dominating assays include spectrometry [1–3], fluorimetry [4–8], chemiluminometry [9–11] and electrochemical [12–19] methods, alone, or preceded by liquid chromatographic [20–22] or FIA [23–25] procedures.

Despite the fact that spectrometry and liquid chromatography have been established as official methods for assaying Ch-related pharmaceutical formulations, e.g. [26, 27], there is surprisingly little information on new methods which might also be useful in routine monitoring. Thus, just a few reports on the use of enzyme sensors [12, 15, 25] have appeared recently, but, sensitive chemiluminometry, for example, has not so far been applied to this field.

Chemiluminometric analysis of Ach/Ch is generally conducted in sample pairs. One sample is exposed to the hydrolysis step by acetylcholine esterase (AchE), the other is kept intact. AchE converts Ach to Ch and in the presence of choline oxidase (ChO), total Ch is oxidized to betaine and H_2O_2. The latter oxidizes luminol to aminophthalate in the presence of horseradish peroxidase (HRP) with emission of light. This enables the simultaneous monitoring of Ach and Ch. The Ch content is directly proportional to the intensity of light emitted in the absence of the hydrolysis step. The Ach content is calculated from the difference in light emission before and after the hydrolysis of Ach. This approach has gained widespread acceptance in many laboratories during the last decade [28–31, 9–11, 24] preferentially being used in analysis of biological samples. The present work introduces it also into pharmaceutical practice. The basic features of the new method, applicable in assaying Ch-related pharmaceutical substances, are the sensitive chemiluminometry of Ch-moiety in conjunction with simple dot-blot technique.

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Experimental

Materials

Unless otherwise stated analytical-reagent grade chemicals were used, as well as doubly distilled water, produced by an all-glass apparatus. ECL western blotting detection reagents, Hyperfilm™-ECL (high performance luminescence detection film), and Hybond™-N Nylon (0.45 μm) membrane were products of Amersham (Amersham, UK).

Phosphate buffer, pH 8.6 (PB). Prepared as 0.1 M $Na_2HPO_4 \times 7H_2O$, and pH adjusted with 85% *o*-phosphoric acid and/or 2 M NaOH.

ChCl and AchCl standards. Choline chloride and acetylcholine chloride were products of Sigma (St Louis, MO, USA).

Stock standards, for both ChCl and AchCl, were prepared as 1×10^{-2} g ml^{-1} solutions in PB or in corresponding matrix-matching medium. Serial dilutions were for both analytes prepared with PB in the concentration range 10 pmol μl^{-1} to 7 nmol μl^{-1} (1.2–812 ng μl^{-1}).

Stock standard solutions were stored at +4°C protected from light.

Enzymes. Enzymes used were: (i) ChO (from *Arthrobacter globiformis*, EC 1.1.3.17, Sigma): stock solution 50 units ml^{-1}, working solution 25 units ml^{-1}; (ii) AchE (from Electric eel, EC 3.1.1.7, type V–S, Sigma): stock solution 500 units ml^{-1}, working solution 83.3 units ml^{-1}; (iii) HRP (EC 1.11.1.7, type VI, Sigma): stock solution 10.0 mg ml^{-1} i.e. 3100 units ml^{-1}, working solution 5.0 mg ml^{-1}.

Stock solutions were aliquoted into 50 μl aliquots and stored at −20°C. They were replenished after 1 week. Working solutions were prepared fresh daily and kept at +4°C during use.

All solutions were prepared in PB and protected from light.

Reaction detection mixture (RDM). This was prepared 15–30 min before the experiment, and protected from light.

It was prepared by mixing 50 μl PB, 10 μl ChO, 25 units ml^{-1}, 2 μl HRP, 5.0 mg ml^{-1} and 50 μl of ECL western blotting reagents mixture (1 + 1), followed by vigorous stirring for at least 15 min. (With fresh stock solutions of ChO stirring should be prolonged to 30 min, otherwise high blank signals may appear.)

Final concentrations of ChO and HRP in RDM were 2.2×10^{-3} units μl^{-1} and 8.9×10^{-2} μg μl^{-1}, respectively.

USP Reference Standards. USP RSs were purchased from The United States Pharmacopeial Convention, Inc, Rockville, MD, USA.

Stock solutions were prepared at concentration levels of original pharmaceutical preparations, i.e. as follows: USP Succinylcholine Chloride RS, G-1, as 2×10^{-2} g ml^{-1} in PB, while USP Oxtriphylline RS, F–1, and USP Acetylcholine Chloride RS, F–1, as 1×10^{-2} g ml^{-1} solutions in corresponding matrix-matching media.

Dilutions were prepared in PB and examined by the same protocols as the corresponding pharmaceutical formulations.

Pharmaceutical preparations. The assayed pharmaceutical preparations were: Quelicin Chloride Injection (Succinylcholine Chloride Injection USP, 20 mg ml^{-1}, 5-ml vials, Abbott Laboratories Ltd, Montréal, Quebec, Canada), Choledyl 200 tablets (200 mg oxtriphylline per tablet, Novopharm, Scarborough, Ontario, Canada), and Miochol (Acetylcholine Chloride for Ophthalmic Solution USP 20 mg and mannitol 60 mg per 2 ml vial, Ivlab Pharmaceuticals, Peterborough, Ontario, Canada).

PB was the diluent for all pharmaceutical preparations.

Matrix-matching media. They served for simulating complex matrices of pharmaceutical preparations in standard solutions for calibration and recovery experiments, in USP AchC1 RS and USP Oxtriphylline RS solutions in accuracy experiments, and as 'field blanks' for evaluation of limit of detection.

Matrix-matching media were: (i) 55.35 mM solution of succinate ion, matching Quelicin injection system corresponding to 110.71 nmol μl^{-1} ChCl; (ii) 17.70 mg ml^{-1} tablet coating alone or with 25.78 mM theophylline, matching Choledyl 200 tablets system (25 mg ml^{-1}) corresponding to 7.31×10^{-3} g ml^{-1} oxtriphylline, i.e. 25.78 nmol μl^{-1} ChCl.

Tablet coating was carefully scraped off several Choledyl 200 tablets, extracted with PB

for 1 h and centrifuged as described for the whole tablets. Before use, the supernatant was checked to be free of any traces of oxtriphylline from the tablet core; (iii) 164.68 mM mannitol, matching Miochol solution system corresponding to 55.05 nmol μl^{-1} AchCl.

All matrix-matching media were prepared in PB.

Apparatus

Quantitation of analytical signals was run on Imaging Research, Inc, MCID M1 System image analyser.

Accumet pH-meter 915 of Fisher Scientific with a combination glass electrode from the same manufacturer was used for pH-readings.

Centrifugations were done on refrigerating centrifuge Eppendorf 5402.

One μl aliquots were applied using Eppendorf Ultra Micro Digital Pipette 4710.

Analytical procedures

Assay of Ch/Ach

One μl of the sample solution was dotted onto the Hybond–N Nylon membrane using the template. One μl of reaction detection mixture (RDM) was applied onto the same dot under protected-from-light conditions. The ECL film was immediately exposed to the luminescing dots. After 20 min exposure, film treatment was performed by developing, washing in water, fixing and final washing in water for 1, 1, 2 and 1 min, respectively.

Evaluation of analytical signals, i.e. relative optical density values (ROD), was run by area densitometric quantitation of spots on the film, in the circle area mode.

For assaying Ach or total of Ach and Ch prior to analysis an appropriate volume of AchE solution (83.3 units ml^{-1}) was added to the sample such as to achieve final activity of 1.1×10^{-2} units μl^{-1} (test I). If interested in Ch content only the sample should be analysed omitting the hydrolysis step (test II). The content of Ach is obtained by the difference.

The alternate dotting of standards and test samples onto the membrane is advised. Timing starts after spotting the first dot.

Figure 1 represents a typical recording on ECL film.

Assays and storage of pharmaceutical preparations

(i) Quelicin injection. Eight ampoules were pooled and divided into individual portions. Each sample was diluted with PB, sealed, heated in a boiling water bath for 10 min, cooled and the content of succinylcholine chloride was determined by test II.

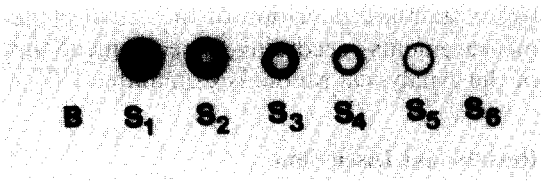

Figure 1
Typical recording on Hybond–N membrane (1:1). B-blank, S_1–S_6-ChCl standard solutions in PB: S_1-710.43; S_2-355.22; S_3-142.09; S_4-71.04; S_5-35.52; S_6-14.21 pmol μl^{-1}.

The pooled sample was stored at +4°C and protected from light.

(ii) Choledyl 200 tablets. Twenty five tablets (average tablet mass 684.48 mg) were crushed, finely powdered and the triturate divided into 25 mg batches. Each sample, accurately weighed, was extracted with 1 ml PB with shaking for 1 h, and centrifuged (1500 rpm, 10 min, 20°C). The supernatant was diluted with PB and oxtriphylline content determined through test II.

Triturated powder was stored at +4°C protected from light and moisture.

(iii) Miochol solution. Six freshly reconstituted vials were pooled and the pool divided into individual samples. From each sample two dilutions with PB were prepared: one for determination of ChCl by test II and the other for determination of AchCl + ChCl by test I. To avoid any excessive hydrolysis of AchCl to ChCl analyses were run immediately.

Estimation of dry mass content

Water in USP RSs was estimated according to USP XXII [26a–c]. Moisture content in Choledyl 200 tablets triturate was determined gravimetrically after drying 4 h at +80°C, and in ChCl and AchCl reagents after drying for 3 h at 105°C.

Evaluation of analytical results

Analytical data are presented as confidence intervals: $CI = \bar{x} \pm t\sigma/n^{1/2}$, at the stated probability level $P(\bar{x}$ = mean value, t = tabulated t-value, σ = standard deviation and n = number of results).

Limit of detection (LOD) is expressed as the concentration derived from: $LOD = f(3\sigma_b)$

(σ_b = standard deviation of the "field blank" measurements) according to recommendation of the Analytical Methods Committee [32].

Results and Discussion

The principle of basing the chemiluminometric analysis of Ch-related substances on Ch content has so far been applied to Ach. However, this proposal also introduces this principle into analysis of succinylcholine chloride and to choline theophyllinate (oxtriphylline). The quantitative liberation of Ch-moiety from succinylcholine chloride was accomplished by alkaline hydrolysis at elevated temperature. In the oxtriphylline molecule, however, there is no disturbance of theophyllinate anion; accordingly Ch-moiety was directly available for the reaction. This means the possible alternative to the USP recommendation for determination of oxtriphylline based on content of theophylline [26b].

For the sake of fast screening of Ach- and Ch-containing preparations simultaneous measurement of several samples is suggested. To reduce deterioration in precision the time of signal measurement, i.e. film-exposure time, should be prolonged up to 20 min, when a plateau of a dynamic response curve is attained (Fig. 2). We found that under these circumstances an acceptable constancy of analytical signals holds within 30 s of an application-to-measurement interval. Namely, the ratios of analytical signals of each preceding dot to the last dot in a series, gave the value of 1.024 ± 0.066 ($\bar{x} \pm \sigma$, $n = 19$), in the concentration range of ChCl of 40–150 pmol μl^{-1}. Practically this means that 8–10 samples could reliably be analysed simultaneously.

Analytical performances and internal quality control

High conformity of calibration lines for ChCl/AchCl standards in PB in matrix-matching media, together with recoveries (Table 1) are confirmation of the method's selectivity and make the use of simple ChCl standards in PB sufficient for all three analytes. This was substantiated with results for PB-standards and matrix-method standards treated together; correlation coefficient and analysis of y-residuals pointed at appropriate linear fit in the concentration range of 40–300 pmol μl^{-1}. Total calibration data included ChCl standards in PB, in Quelicin-matched matrix and in Choledyl-matched matrix, $n = 7(6)$, and AchCl standards in PB and in Miochol-matched matrix, $n = 6\,(5)$ (number of parallels in parentheses). For ROD vs −log ChCl/AchCl concentration (mol μl^{-1}) the following regression line was estimated ($n = 13$): slope, $b(\sigma) = -1.16\ (0.020)$; intercept, $a(\sigma) = 12.13$ (0.203); correlation coefficient, $r = 0.9983$.

The method's internal quality control (Table 2) represents excellent accuracy for all analytes.

Precision studies were completed by estimating day-to-day RSD values (Tables 2–5). Precision appears to be independent of the run type. Introduction of exact correction factors

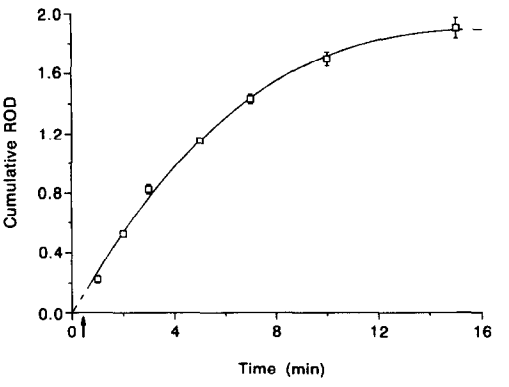

Figure 2
Dynamic response curve for 353.50 pmol μl^{-1} ChCl on Hybond–N membrane. Individual points refer to mean ROD ± σ for five replicates. The arrow indicates the start of film exposure.

Table 1
Analytical performances of the method

Analytical system	Calibration sensitivity (μl pmol^{-1})*†	Recovery (%)	Limit of detection (pmol μl^{-1})
ChCl/AchCl stds in PB	−1.808 ± 0.118 (11)	—	37.05
ChCl stds in Quelicin matrix	−1.834 ± 0.072 (8)	101.88	37.10
ChCl stds in Choledyl 200 matrix	−1.751 ± 0.099 (7)	97.26	38.98
AchCl stds in Miochol matrix	−1.782 ± 0.093 (5)	98.94	36.94

*$\bar{x} \pm \sigma$.
†Number of parallels in parentheses.

Table 2
Internal quality control of the method (mg analyte found in 10.00 mg USP RS analysed)

Analyte* (USP RS)	CI†‡§ (mg)	RSD (%)	Accuracy (%)
Succinylcholine chloride (USP Succinylcholine Chloride RS, G-1)	9.91 ± 0.35 (19)	4.8	−0.9
Oxtriphylline (USP Oxtriphylline RS, F-1)	9.81 ± 0.34 (18)	4.5	−1.9
Acetylcholine chloride (USP Acetylcholine Chloride RS, F-1)	9.98 ± 0.48 (19)	6.6	−0.2

*Accuracy testing performed at four concentration levels corresponding with 47–170 pmol μl^{-1} ChCl.
†$P = 0.995$.
‡Number of individual samples in parentheses; at least three parallels of each sample.
§Calculated on the dried mass basis.

Table 3
Analysis of Quelicin injections*

Performance characteristic	CI†‡ (nmol μl^{-1})		σ (nmol μl^{-1})	SEM (nmol μl^{-1})	RSD
Type of anhydrous succinylcholine chloride assay§	(mg ml^{-1})		(mg ml^{-1})	(mg ml^{-1})	(%)
Within a day	56.39 ± 2.74 / 20.38 ± 0.99	(10)	2.35 / 0.85	0.74 / 0.27	4.2
Day to day	55.91 ± 2.01 / 20.20 ± 0.73	(16)	2.45 / 0.88	0.61 / 0.22	4.4

*Injections stated to contain 20 mg ml^{-1} anhydrous succinylcholine chloride USP: USP requirement: 90.0–110.0% of the labeled amount of anhydrous succinylcholine chloride [26a].
†$P = 0.995$.
‡Number of individual samples in parentheses; each sample analysed at least in triplicate.
§Final dilution corresponding to ChCl-standard level of 94 pmol μl^{-1}.

for each dot or individual measurement of samples (eventually with reduced exposure time) could be recommended in order to achieve maximum precision.

Nevertheless, in regard to figures of merit (Tables 1 and 2) the method proved to be superior or comparable to some enzyme sensors [12–16, 25], spectrometric [1–3] and luminometric [24] methods. Due to favourable LOD-values the method might also be applicable to analysis of biological samples.

In addition to Hybond–N Nylon membrane, some other nucleic acid transfer blotting media (e.g. Bio-Rad Trans Blot, Bio-Rad Immun-Lite, Pall Biodyne, Zeta Probe) and even Whatman No. 1 qualitative filter paper could successfully be used, but with several fold decreased detection sensitivity.

Analysis of pharmaceutical preparations

In addition to highly accurate analysis of USP Succinylcholine Chloride RS, the results of Quelicin injection analysis (Table 3) proved once again that the hydrolytic cleavage of the ester bond in succinylcholine chloride takes place quantitatively by heating in phosphate buffer pH 8.6.

The method was also evidenced as convenient for analysis of oxtriphylline even in tablets (Table 4). Contrary to the USP requirement [26b] to estimate oxtriphylline content through theophylline, an alternative way of estimation based on determination of Ch-moiety is offered.

During the course of preliminary studies the method proved to be a reliable means of assaying the mixtures containing various proportions of ChCl/AchCl (Fig. 3). Results obtained for Miochol intraocular solution (Table 5) demonstrated that even freshly reconstituted solutions suffer from contamination by ChCl of about 2.9%, calculated on the mole basis.

Table 4
Analysis of Choledyl 200 tablets*

Performance characteristic	CI†‡ (μmol per 25.00 mg triturate)§	σ (μmol per 25.00 mg triturate)§	SEM (μmol per 25.00 mg triturate)§	RSD
Type of oxtriphylline assay‖	(mg per tablet)	(mg per tablet)	(mg ml per tablet)	(%)
Within a day	$\frac{24.83 \pm 2.59}{190.29 \pm 19.87}$ (7)	$\frac{1.59}{12.18}$	$\frac{0.60}{4.60}$	6.4
Day to day	$\frac{24.76 \pm 1.58}{189.22 \pm 12.09}$ (12)	$\frac{1.56}{11.97}$	$\frac{0.45}{3.46}$	6.3

*Tablet stated to contain 200 mg oxtriphylline USP: USP requirement: 90.0–110.0% of the labeled amount of anhydrous theophylline [26b].
†$P = 0.995$.
‡Number of individual samples in parentheses; four parallels of each sample.
§Calculated on the dried basis.
‖Final dilution corresponding to ChCl-standard level of 78 pmol μl^{-1}.

Table 5
Analysis of Miochol intraocular solution*

Performance characteristic	CI†‡ (nmol μl^{-1})	σ (nmol μl^{-1})	SEM (nmol μl^{-1})	RSD
Analyte(s)§	(mg per 2 ml)	(mg per 2 ml)	(mg per 2 ml)	(%)
AchCl + ChCl	59.60 ± 4.21 / 21.51 ± 1.53 (7)	2.58 / 0.93	0.98 / 0.35	4.3
ChCl	1.71 ± 0.19 / 0.48 ± 0.05 (7)	0.12 / 0.03	0.04 / 0.01	6.8
AchCl	57.89 ± 4.15 / 21.03 ± 1.51 (7)	2.55 / 0.92	0.96 / 0.35	4.4

*2-ml vial of Miochol stated to contain 20 mg AchCl USP and 60 mg mannitol; USP requirement: 90.0–115.0% of the labeled amount of AchCl [26c].
†$P = 0.995$.
‡Number of individual samples in parentheses; each sample analysed at least in triplicate.
§Pool dilutions corresponding to ChCl-level of 85 and 57 pmol μl^{-1} in test I and test II, respectively.

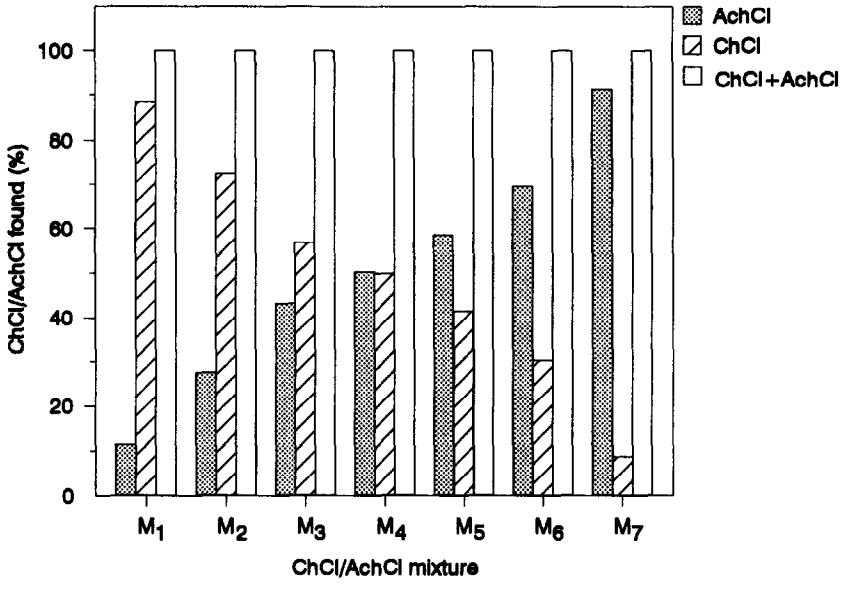

Figure 3
Analyses of ChCl/AchCl mixtures. Molar proportions of ChCl and AchCl in mixtures M_i to M_7 are 9.0 + 1.0, 7.0 + 3.0, 6.0 + 4.0, 5.0 + 5.0, 4.0 + 6.0, 3.0 + 7.0 and 1.0 + 9.0, respectively. Total ChCl + AchCl concentration in M_1–M_7 fixed to level of 306 pmol μl^{-1}. Means of triplicate for each mixture presented.

Conclusion

The basic features of the present study could be summarized through the following points: (1) Development of a new, simple, sensitive and selective method for assaying succinylcholine chloride, choline theophyllinate, and AchCl, alone or in a mixture with ChCl, based on dot-blot chemiluminometry of Ch-moiety. (2) Introduction of a new principle of quantitative analysis of succinylcholine chloride and choline theophyllinate, after alkaline hydrolysis and without any pretreatment, respectively. (3) The method prospectively appears to be applicable to the determination of some other Ch-related pharmaceutical substances.

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