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TECHNICAL CONSIDERATIONS IN THE GAS CHROMATOGRAPHIC ANALYSIS OF DESIPRAMINE

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SUMMARY

Procedures are presented for minimizing variability in and interferences with the gas chromatographic determination of desipramine in plasma. Careful consideration of procedures for sample collection and storage, drug separation from matrix components, and chromatography appears to be a prerequisite for avoiding inaccurate and imprecise determinations of this antidepressant, especially at levels below 20 μg/l. Numerous pitfalls are examined and optimal conditions for obviating them are presented.

INTRODUCTION

The determination of tricyclic antidepressant levels in biological specimens has received much attention in recent years. These levels have been utilized in studies of product bioavailability, patient compliance, toxic overdose, and in relating dose or plasma level to clinical response. The validity of conclusions drawn from such studies depends heavily upon the accuracy of the drug level determinations utilized.

Numerous assay techniques for the tricyclics have been described [1—6]. Several popular procedures utilize gas—liquid chromatography with nitrogen-specific detection [7—12]. This technique is relatively inexpensive, offers adequate sensitivity in concentration ranges encountered during clinical use of the tricyclics, and is adequate for many single-dose pharmacokinetic studies where plasma levels as low as a few micrograms per liter may be encountered.

Attempts to implement published nitrogen-detector gas chromatographic assay procedures for the tricyclic antidepressant desipramine in our laboratory have met with a variety of difficulties. Drug adsorption onto glassware and column materials has led to decreased sensitivity, non-linear standard curves and unacceptable reproducibility. As a result of the nitrogen detector's high

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sensitivity to certain nitrogenous compounds, extraneous peaks from biological and chemical contaminants have complicated analyses at low drug concentrations. Our objective in undertaking the present study was to identify and isolate sources of variability and interference and develop a gas chromatographic method for accurately and reproducibly determining desipramine at levels approaching 1 μg/l in as small a plasma sample as possible.

This report systematically presents observations on the various aspects of a viable chromatographic assay for the tricyclics. It considers each step in the analysis in turn and provides negative as well as positive findings in an attempt to assist readers who may encounter one or more technical difficulties in the course of implementing and/or routinely performing such assays. It concludes with a presentation of those conditions found to be most satisfactory and their application to drug level determinations in human volunteers.

METHODS AND RESULTS

Reagents and chemicals

Methanol, hexane and *n*-butanol were purchased distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and were used without further purification. Isoamyl alcohol (Mallinckrodt, St. Louis, MO, U.S.A.) was redistilled in glass (b.p. 128.5°C). Water was double-distilled in glass and washed with hexane before use in extractions. Desipramine·HCl and amitriptyline·HCl were gifts of USV Pharmaceutical Corp., Tuckahoe, NY, U.S.A. and Merck, Sharp and Dohme, West Point, PA, U.S.A., respectively. [^{3}H]Desipramine (38.68 Ci/mmole) was a 7.7 μg/l solution in ethanol (New England Nuclear, Boston, MA, U.S.A.). All other reagents were analytical reagent grade and were used without further purification.

General approach

A number of techniques are available for isolating tricyclics from plasma*. The basic approach consists of extraction with an organic solvent at an alkaline pH, back-extraction into acid to remove interfering biological components and realkalization. The drug is then prepared for injection by either extracting into a small volume of organic solvent or extracting with a larger volume followed by evaporation and subsequent reconstitution with organic solvent. Typically a tricyclic of minimal therapeutic relevance to the study at hand is used as an internal standard.

Sample collection and storage

Adsorptive losses of desipramine must be considered whenever the drug is placed in contact with glass surfaces, especially in the low μg/l concentration range. These losses are most frequently overlooked, not in the assay procedure itself, but rather in the collection and storage of patient samples and during the preparation of serum or plasma standards.

Vacutainer® tubes must be avoided because of their well-documented

*Serum and heparinized plasma behaved identically in our hands and will be used synonymously throughout this report. EDTA and oxalate produced interfering peaks and were not suitable as anticoagulants.

plasticizer interferences [13]. The plasticizer, tris(2-butoxyethyl)phosphate, interferes with the binding of the tricyclics to α_1-acid glycoprotein, thereby effecting a redistribution of the drug into erythrocytes. The apparent plasma level of the drug is thus altered and the plasticizer must, therefore, be avoided even when it produces no chromatographic interference per se. Our samples were drawn into plastic syringes. The needle was then removed and the syringe emptied into a silanized and heparinized glass tube. The separated plasma was immediately frozen at −20°C in a silanized glass tube and not permitted to thaw until assay. Once thawed, we found significant drug losses at concentrations of less than 20 μg/l in as little as 3 h at 25°C.

Preparation of standards

In the course of establishing recoveries of drug from plasma at low concentrations, discrepancies were noted which led us to evaluate our procedure for the addition of known quantities of drug. Recovery and reproducibility appeared to depend upon the solvent utilized to prepare drug stock solutions. Serum standards were therefore prepared using desipramine stock solutions in methanol, water and serum. Of these, water was judged most acceptable. Inaccurate pipetting of small volumes of serum resulted in poor reproducibility. When methanol was used as the "spiking" solution vehicle, reproducibility was also diminished. This may have been due to a localized protein precipitation observed upon addition of the methanolic solution, an effect not observed with the other vehicles.

To determine whether stock solution solvent affects adsorptive losses in the standards, thereby decreasing recoveries, a solution of [^{3}H]desipramine was prepared in each of the three aforementioned solvents. These stock solutions were then used to prepare serum samples at a concentration of 20 μg/l. Twenty-five microliters of stock solution were added, with constant stirring, to 2.5 ml of serum in a narrow, cylindrical silanized glass tube. The geometry was such that sampling could be achieved while maintaining an essentially constant surface area/volume ratio. Samples were withdrawn for analysis at 5 min intervals over a period of about 60 min. All specimens appeared stable over this relatively brief time period and adsorption was ruled out as the source of variable recoveries.

As a result of these findings, all subsequent studies were performed using a 1 g/l primary stock solution of desipramine hydrochloride in water. This solution is stable for at least three months at 4°C. At the time of assay a 2 mg/l secondary stock solution is prepared from which plasma standards are then derived.

Internal standard

Amitriptyline was chosen as the internal standard for our assay, but other tertiary tricyclics, such as imipramine, were also suitable. Protriptyline was evaluated because of its use by other authors, but it produced unacceptable peak shapes under our chromatographic conditions. An aqueous solution (600 μg/l) of internal standard was added to the serum sample prior to the first extraction step. More reproducible results were obtained by this method than by the addition of internal standard to the first extraction solvent.

Sample size

In selecting a sample for extraction, our objective was to minimize the sample size while ensuring adequate volume for repeated measurements. Sample sizes of 0.5—3.0 ml were evaluated. The smaller volumes limited sensitivity while the larger sample sizes yielded disproportionately large contaminant peaks on the chromatogram. Samples of 1 ml seemed to provide sufficient sampling accuracy and adequate sensitivity with minimal interferences.

Sample cleanup

Because serum and plasma contain numerous interfering nitrogenous components, the original plasma was washed with hexane as were subsequent aqueous phases of the sample workup. While this failed to remove all extraneous peaks, it did eliminate all interferences at the retention times of interest.

Alkalinization

Since desipramine is a weak base ($pK_a = 10.2$), at higher pH the drug is more extractable and recoveries are higher. Specifically, recovery from plasma increased from 35% at pH 9.0 to 65% at pH 11.5. Unfortunately, more alkaline conditions increased the extraction of interfering serum constituents as well. Alkalinization with ammonium hydroxide to a sample pH of 11.5 provided adequate recovery with minimal contamination.

Extraction

Various extraction solvents were evaluated using a variety of phase volume ratios and extraction times. Hexane containing a small percentage of isoamyl alcohol was found most efficient. Systematically increasing the alcohol content from 2 to 10% produced only slight improvement in recovery. As the alcohol content increased, gel formation at the interface began to interfere with phase separation. Interfering peaks also became more pronounced. Identical recoveries were subsequently obtained when isoamyl alcohol was replaced by *n*-butanol. Glass-distilled *n*-butanol could be used as purchased whereas redistillation of commercial isoamyl alcohol was deemed essential. A hexane—*n*-butanol (98:2) extraction solvent was, therefore, selected.

Extraction efficiency plateaued at a phase volume ratio of 1:1, with higher volumes of extractant yielding little improvement. Equilibration times were a function of the agitation imposed on the system. Samples vortexed at a high speed equilibrated within 3 min, while samples placed in a mechanical shaker (Eberbach Corp., Ann Arbor, MI, U.S.A.) at 200 oscillations per min required at least 15 min.

Back-extraction

Insignificant differences were noted among phosphoric, hydrochloric and sulfuric acids when each was utilized for the back-extraction of tricyclic into acid, and 0.1 *N* H_2SO_4 was selected.

Concentration of final extract

Two methods were considered for achieving a final concentrated tricyclic

extract suitable for injection. Extraction into volumes of hexane–butanol ranging from 50 to 200 μl were evaluated. Volumes of less than 100 μl resulted in poor extraction efficiency because of unfavorable phase volume ratios. Above 100 μl sensitivity was limited by our 10 μl injection volume which dictated that less than 10% of extracted drug could be placed on the column using this method. Alternatively, the final extraction was performed with a more favorable phase ratio of about 1:1 followed by subsequent evaporation of the extractant and reconstitution into a small volume of methanol prior to injection. This procedure worked well as long as precautions were taken to avoid several potential sources of adsorptive loss during the evaporation step.

Adsorptive losses during evaporation

Large decreases in recovery were observed when samples were evaporated from glass surfaces (Reacti-Vials, Pierce, Rockford, IL, U.S.A.). The observation of a proportionality between evaporative surface area and drug loss is consistent with adsorption onto the glass surface. Neither the use of polypropylene or polystyrene containers nor the addition of *n*-butanol, isoamyl alcohol or glacial acetic acid to the evaporating solution alleviated the problem. Silanization (Siliclad, Clay Adams, Parsippany, NJ, U.S.A.) reduced adsorption, and silanized glassware was utilized for collection, storage, and workup of samples. Such glassware was not useful in the evaporative step, however, because a contaminant peak with a retention time identical to that of desipramine appeared on the chromatogram. Instead, the glass vials were cleaned by washing in a non-phosphate detergent (Labtone, VWR Scientific, Boston, MA, U.S.A.), soaking consecutively in chromic acid and 10% NaOH and then sonicating. To deactivate adsorption sites, vials were then soaked in 20% triethylamine in methanol and rinsed consecutively with methanol and hexane–*n*-butanol (98:2) to remove excess amine. Using this procedure, little adsorptive loss occurred during the evaporation step and assay sensitivity and reproducibility were ensured.

Chromatographic considerations

A Varian Model 3740 gas chromatograph equipped with nitrogen/phosphorus detector (Varian, Palo Alto, CA, U.S.A.) was used. The carrier gas flow-rate and oven temperature (see below) were adjusted to provide maximum resolution of serum components from the peaks of interest. Oven temperature was elevated after the peaks of interest eluted to clean the column between injections. The detector and injector temperatures were maintained as high as drug stability would permit to prevent accumulation of sample components at these locations. The silanized glass-wool plug at the head of the column was replaced every 30 injections, a procedure found essential if reproducible peak shapes and retention times were to be achieved.

Initially, separations were performed using 3% OV-17 on Gas-Chrom Q, 100–200 mesh (Applied Science Labs., State College, PA, U.S.A.). Peak tailing and variable peak shape, probably due to adsorption, rendered this packing unacceptable. "Doping" the column with a concentrated drug solution prior to the analysis did reduce drug adsorption, but it was effective only for short and quite variable periods of time. A commercially available clinical packing, 3%

SP-2250 on 80—100 Supelcoport (Supelco, Bellefonte, PA, U.S.A.), advertised as specially deactivated toward tricyclics, was found to minimally adsorb desipramine and not to require "doping".

Derivatizations with acetic and trifluoroacetic anhydrides were attempted in an effort to improve response. Although detector response was modestly increased, the increase was not sufficiently large to offset the fact that extraneous peaks also increased in magnitude and number to the point where interference became a problem. Consequently, derivatization was abandoned.

Optimal assay procedure

After due consideration of the aforementioned, the following assay procedure was adopted. One milliliter of plasma or serum is pipetted into a 10-ml silanized glass tube fitted with an extraction tube plug (Oxford Laboratories, Foster City, CA, U.S.A.). Twenty microliters of an aqueous internal standard solution, containing 12 ng of amitriptyline·HCl, are added. The sample is acidified with 200 μl of 1 *N* HCl, washed for 1 min with 1 ml of hexane, centrifuged (1686 *g*) for 5 min, and the upper organic phase discarded. The aqueous phase is then alkalinized with 300 μl of concentrated ammonium hydroxide and shaken mechanically for 15 min with 1 ml of hexane—*n*-butanol (98:2). The upper phase is transferred to a clean 10-ml silanized glass tube and 1 ml of 0.1 *N* H_2SO_4 added. The tube is shaken mechanically for 15 min, centrifuged, and the upper organic phase discarded. The acidic aqueous phase is washed with 2 ml of hexane (5 min agitation), transferred to a 10-ml silanized glass tube, alkalinized with 100 μl of ammonium hydroxide, and extracted for 15 min with 1.5 ml of hexane—*n*-butanol (98:2). The organic phase is transferred to a clean, deactivated Reacti-Vial® and evaporated under a stream of nitrogen at 25°C. It should be noted that evaporated samples stored overnight at either 25°C or −20°C display unacceptable variability. If the assay is to be interrupted, samples should be stored just prior to the evaporation step. The residue is reconstituted with 20 μl of methanol and 6 μl are chromatographed under the following conditions: column temperature 243°C, injector 300°C, detector 300°C, carrier gas (ultra high purity nitrogen) 17 ml/min, air 175 ml/min, and hydrogen 4.5 ml/min. Rubidium bead current is set according to manufacturer's recommendations. Plasma concentrations are determined by comparison of peak height ratios (desipramine/amitriptyline) to comparable ratios for extracted standards.

Standard curves

Desipramine serum standards ranging from 5 to 200 μg/l were prepared using the aqueous stock solutions discussed above. Peak height ratios and concentrations were linearly related over this concentration range. The slopes and intercepts of five standard curves obtained over a period of about two months were compared in an analysis of variance [14] in order to assess their variation with time. The data (Table I) indicate that neither the intercepts nor the slopes of the standard curves varied with time ($p > 0.05$). We interpret this to mean that changes in column performance were not significant over the time span considered and matrix components present in the various lots of serum used to prepare the standards did not introduce significant variability.

TABLE I

ANALYSIS OF VARIANCE ON SLOPES AND INTERCEPTS DERIVED FROM STANDARD CURVES FOR DESIPRAMINE IN PLASMA

F statistic (slope) (4,14) = 0.176 N.S. ($p > 0.05$); F statistic (intercept) (4,14) = 0.014 N.S. ($p > 0.05$).

Curve No.	r^2	Slope	Variance of slope	Intercept	Variance of intercept
1	0.9947	0.03267	2.18×10^{-6}	—0.1128	0.00716
2	0.9961	0.03322	4.43×10^{-6}	—0.0764	0.01840
3	0.9861	0.03382	8.16×10^{-6}	—0.0597	0.02600
4	0.9924	0.03398	8.73×10^{-6}	—0.0833	0.02800
5	0.9852	0.03106	1.46×10^{-5}	—0.0879	0.04700

Reproducibility

Six replicate analyses of the same 5 μg/l serum sample on a single day yielded a coefficient of variation of 5.3%. Six measurements of the same 50 μg/l sample over a period of one month yielded a between-day coefficient of variation of 6%. Analytical recovery from plasma or serum was 65% as determined by comparison of peak heights from extracted samples with those from an equivalent amount of drug in methanolic solution injected directly. This was in contrast to an 80% recovery from water. Extraction efficiency was also diminished by prolonged storage (—20°C, 3 months or more) of either the serum used to prepare standards or the patient samples themselves. Other than speculating that plasma protein binding may somehow account for the discrepancies, the reasons for these observations remain obscure.

APPLICATION TO PATIENT SPECIMENS

In order to evaluate the assay procedure on actual plasma samples, a single

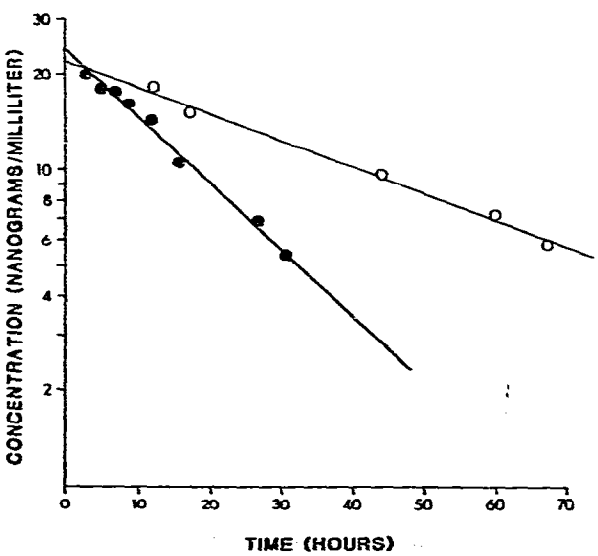

Fig. 1. Log plasma concentration versus time curves for desipramine following single 75-mg oral doses to young (●) and elderly (○) volunteers.

75-mg oral dose of desipramine·HCl (Norpramin, Merrell-National, Cincinnati, OH, U.S.A.) was administered to both a young (29 years) normal volunteer and an elderly (67 years) depressed patient. Plasma levels of desipramine over time are shown in Fig. 1. Postabsorptive levels declined monoexponentially with apparent half-lives of 14.7 and 36.5 h in the young and elderly subjects, respectively. The assay is currently being utilized to perform pharmacokinetic studies on depressed patients following both single and multiple oral doses. Our plasma level versus time curves have remained log-linear down to desipramine concentrations of 1 $\mu g/l$, confirming our estimate of that level as our lower level of quantitative sensitivity.

DISCUSSION

Many studies have been conducted in an attempt to relate clinical response to tricyclic therapy to plasma levels. In addition, several pharmacokinetic studies have been undertaken following both single and multiple oral doses [15, 16] and others are certain to follow. It has recently become clear that sample collection techniques may have influenced the results of a number of these reports. For example, the use of Vacutainers® causes the inadvertent introduction of tris(2-butoxyethyl)phosphate, a plasticizer present in the stoppers, which displaces basic drugs from binding sites of α_1-acid glycoprotein, thus enhancing diffusion of drug into erythrocytes and reducing plasma levels. The present report illuminates several other areas where inaccuracies may have gone unnoticed in previous studies. Consideration must be given to careful removal of contaminants and adsorptive losses during sample storage, workup and chromatography. The significance of these contributing factors will increase dramatically as one approaches the "nanogram barrier" during low-dose pharmacokinetic studies of these heavily tissue-distributed drugs. Hopefully, the results reported herein will assist analysts in avoiding the numerous pitfalls that confront them during the analysis of the tricyclic antidepressants, especially at low concentration.

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