Journal of Chromatography, 212 (1981) 221–228
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,813

LIQUID CHROMATOGRAPHY MEASUREMENT OF CORTISOL IN METHYLENE CHLORIDE EXTRACTS OF AQUEOUS SOLUTIONS

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(Received March 20th, 1981)

SUMMARY

Variable and incomplete analytical recovery of cortisol (*i.e.*, range 59–101 %) was observed from aqueous standards prepared for high-performance liquid chromatography (HPLC) by extraction into methylene chloride, solvent evaporation and residue reconstitution in our mobile phase. Analytical recoveries of cortisol following solvent evaporation in glass tubes without extraction were low (49 %) and showed no improvement by using containers of different composition. Comparison of a radiochromatogram for tritium-labelled cortisol and a chromatogram for unlabelled cortisol following sample preparation for HPLC indicated that low analytical recoveries were not the result of cortisol degradation during sample preparation. Adding polyethylene glycol (mol. wt. 20,000) to methylene chloride before solvent evaporation dramatically improved the analytical recovery of cortisol.

INTRODUCTION

Numerous methods for measuring cortisol in serum and plasma utilizing high-performance liquid chromatography (HPLC) have been reported[1–11] since Wortmann *et al.*[1] described the first method in 1973. Sample preparation, prior to chromatography, is an integral part of the HPLC method and typically consists of extracting the sample with an organic solvent, evaporating the extract to dryness and reconstituting the residue with the HPLC mobile phase. Van den Berg *et al.*[2] have shown that methylene chloride has a higher extraction efficiency for cortisol than either diethyl ether or ethyl acetate and has been the solvent selected by subsequent workers. The low analytical sensitivity of the various methods at physiological concentrations of cortisol requires that reconstitution volumes be substantially smaller than the original sample volume, with previously reported concentration factors (*i.e.*, sample volume/reconstitution volume) ranging from 5- to 333-fold.

While examining the stability and degradation products of cortisol in aqueous solutions using a recently published HPLC method[3], we observed variable and incomplete analytical recovery of cortisol during sample preparation. Garg *et al.*[4] previously observed only 72 % recovery of cortisol from aqueous standards and Scott

and Dixon[5] reported that the analytical recovery of cortisol from 30 g/l bovine albumin was approximately 10% higher than from water (*i.e.*, 76%). Other workers using analogous sample preparation protocols have observed higher recoveries from human sera (*e.g.*, 78%[6], 83%[7] and 96%[3]), but have bypassed potential problems encountered with aqueous standards by either preparing more concentrated standards for direct injection into the instrument without extraction or adding standard to a portion of the serum specimen for simultaneous work-up.

In this study, we examine the analytical recovery of cortisol after solvent evaporation and reconstitution with our mobile phase. The evaporation step is performed in a variety of containers and over a wide range of cortisol concentrations. Radioactively labelled cortisol is used to verify the low analytical recovery and to check for possible degradation of cortisol during sample preparation. Finally, addition of a macromolecular matrix material (*i.e.*, polyethylene glycol, mol. wt. 20,000) to the methylene chloride before solvent evaporation is examined as a technique for improving the analytical recovery and precision of cortisol measurement.

MATERIALS AND METHODS*

Chemicals

Cortisol, Standard Reference Material 921, was from the National Bureau of Standards (NBS) (Washington, DC, U.S.A.). [1,2,6,7-$^3H_4$]cortisol, 1 Ci/l (specific activity 93.1 Ci/mol), was from New England Nuclear (Boston, MA, U.S.A.). Methylene chloride and methanol (both distilled in glass) were from Burdick & Jackson Labs. (Muskegan, MI, U.S.A.). Ethanol (absolute) was from U.S. Ind. Chem. (Tuscola, IL, U.S.A.). Polyethylene glycol (PEG), mol. wt. 20,000, was from Fisher Scientific (Pittsburgh, PA, U.S.A.). Ready-Solv HP scintillation cocktail was from Beckman (Fullerton, CA, U.S.A.). Water (resistivity $\geqslant$ 10 MΩ) was charcoal filtered, deionized and filtered through a membrane of pore size 0.45 μm before use.

Equipment

The chromatographic system included: an SP8000 liquid chromatograph and data system from Spectra-Physics (Santa Clara, CA, U.S.A.); a μBondapak C_{18} reversed-phase column (30 cm $\times$ 3.9 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) and a Varichrom variable wavelength detector from Varian (Palo Alto, CA, U.S.A.). An Isocap 300 liquid scintillation system from Searle Analytic (Des Plaines, IL, U.S.A.) was used for measuring tritium.

Procedures

Extraction. A 1-ml volume of aqueous cortisol solution was extracted with 10 ml of methylene chloride by shaking at 280 strokes per min on a mechanical shaker for 10 min. After shaking, the layers were allowed to separate completely and 8.0 ml of the methylene chloride layer were transferred to a 15-ml glass centrifuge tube for evaporation.

Evaporation. Methylene chloride was evaporated by partially immersing the

* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

centrifuge tube in a water-bath at 40°C under a stream of nitrogen gas. An apparatus constructed of stainless-steel tubing directed the nitrogen flow into the tube, and the gas flow was maintained between 1 and 3 cm above the surface of the methylene chloride during evaporation.

Reconstitution and chromatography. Residues after evaporation were reconstituted by adding 100 μl of the HPLC mobile phase (methanol–water, 55:45, v/v), immediately replacing the container cap and vortexing the solvent over the interior surface of the tube for 15 sec. A 50-μl volume of solution was injected for chromatographic analysis. The mobile phase was pumped at a flow-rate of 1.0 ml/min producing a back pressure of 9 MPa (1300 p.s.i.). Column eluent was monitored at 242 nm at detector settings between 5 and 20 milliabsorbance units full scale.

Recovery studies

Standards: Stock solutions containing 500 μmol/l and 640 μmol/l of cortisol were prepared in absolute ethanol, stored at 4°C, and used within 1 month. Appropriate dilutions of stock solutions were made into methylene chloride, our mobile phase, and water as described in the studies below.

Calculations. Analytical recoveries were calculated by dividing the peak area of absorbance or radioactivity measured in a sample prepared following our protocol (*i.e.*, solvent evaporation and reconstitution) by the peak area of absorbance or radioactivity measured in a standard diluted directly in our mobile phase corresponding to the theoretical concentration of the reconstituted sample (*i.e.*, assuming complete analytical recovery). The dividend was then multiplied by 100.

Concentration effect. Solutions containing 0.04, 0.12, 0.40, 1.20, 4.0 and 8.0 μmol/l of cortisol in methylene chloride were prepared and 8-ml portions transferred to a corresponding centrifuge tube, evaporated to dryness and reconstituted with 100 μl of our mobile phase. We prepared the various concentrations of cortisol by sequentially diluting our 640 μmol/l standard four-fold with methylene chloride and then to the cortisol concentrations used in the study, *e.g.*, a 40-fold dilution to yield a cortisol concentration of 4.0 μmol/l. Chromatographic peak areas for the reconstituted samples were compared to the peak area of a 3.2, 8.0 or 9.6 μmol/l standard prepared by diluting our 640 μmol/l standard with mobile phase. After reconstituting samples with 100 μl of mobile phase, the two solutions with the lowest concentrations of cortisol (*i.e.*, 0.04 and 0.12 μmol/l) were injected directly into the HPLC system and compared to a standard corresponding to complete analytical recovery (*i.e.*, 3.2 and 9.6 μmol/l). Reconstituted solutions from the four remaining samples were diluted further with mobile phase as described in Table I.

Tubes for evaporation. A 40 nmol/l solution of cortisol in methylene chloride was prepared and 8 ml transferred to each of five different types of tubes: a Pyrex brand glass, 15-ml screw-capped centrifuge tube, Corning, (Corning, NY, U.S.A.); a Ray-Sorb, 1.8 × 15 cm, low-actinic glass tube, Kimble Products (Vineland, NJ, U.S.A.); a Falcon No. 2070, 50-ml conical polypropylene tube, Becton-Dickinson (Oxnard, CA, U.S.A.); a Tefzel ETFE (ethylenetetrafluoroethylene), 12-ml tube, Nalgene (Rochester, NY, U.S.A.); and a Pyrex brand glass, 15-ml tube rinsed in a 10 g/l solution of Dow-Corning 360 fluid (Dow Chemical, Midland, MI, U.S.A.), a clear liquid containing dimethyl polysiloxane which dries to a chemically inert, water repellant surface after heating to 300°C for 30 min. The cortisol solution was prepared by

sequentially diluting our 640 μmol/l standard 16,000-fold with methylene chloride, *i.e.*, a 200-fold dilution followed by an 80-fold dilution. The solution was evaporated to dryness and the residue reconstituted with 100 μl of our mobile phase. Chromatographic peak areas measured for the reconstituted specimens were compared to the peak area of a 3.2-μmol/l standard prepared by diluting 50 μl of our 640-μmol/l standard to 10.0 ml with mobile phase.

Tritiated cortisol. A 50-nmol/l solution of cortisol in methylene chloride containing approximately 6.25 μCi/l of tritiated cortisol was prepared and an 8-ml portion was transferred to a glass centrifuge tube, evaporated to dryness and reconstituted with 100 μl of our mobile phase. During chromatography, fractions were collected at the detector outlet into scintillation vials at 30-sec intervals beginning at the time of injection. Then 10 ml of scintillation fluid was dispensed into each vial, thoroughly mixed with each fraction and counted for 10 min.

Polyethylene glycol study. Solutions containing 50 nmol/l of cortisol and various concentrations of polyethylene glycol in methylene chloride were prepared and 8-ml portions transferred to centrifuge tubes, evaporated to dryness and reconstituted with 100 μl of mobile phase. Cortisol was added to the solutions by initially preparing a 100-fold dilution of our 500 μmol/l standard in methylene chloride and then dispensing 100-μl portions into eleven 10-ml volumetric flasks. Methylene chloride and ten solutions containing 0.004, 0.01, 0.10, 0.40, 1.0, 2.0, 5.0, 10, 20 and 50 mg/l of polyethylene glycol in methylene chloride were added to dilute the 100 μl of cortisol solution to 10.0 ml. Chromatographic peak areas for the reconstituted specimens were compared to the peak area of a 4.0-μmol/l standard prepared by diluting 80 μl of our 500-μmol/l standard to 10.0 ml with mobile phase.

Extracted aqueous cortisol. Seven 1-ml aliquots of a 500-nmol/l solution of cortisol in water were each extracted with 10 ml of methylene chloride. The methylene chloride layers were combined, mixed and dispensed in 8-ml portions into seven glass centrifuge tubes. A 160-μl volume of a 1.0-g/l solution of polyethylene glycol in methylene chloride was pipetted into each tube. The extracts were evaporated to dryness, and reconstituted with 100 μl of mobile phase. Chromatographic peak areas for the reconstituted specimens were compared to the peak area of a 4.0-μmol/l standard prepared by diluting 80 μl of our 500-μmol/l standard to 10.0 ml with mobile phase.

RESULTS AND DISCUSSION

In a preliminary study, we observed analytical recoveries ranging between 59 and 101% upon repeated analysis ($n = 6$) of an aqueous solution containing cortisol (500 nmol/l) in which sample preparation consisted of extraction with methylene chloride, solvent evaporation and reconstitution of the residue with the mobile phase. Additional studies in which cortisol standard (50 nmol/l) was dissolved directly in methylene chloride (*i.e.*, bypassing sample extraction) showed analytical recoveries ranging from 47 to 75%. These observations suggested to us that cortisol was either decomposing during sample preparation or was dissolving incompletely from the side of the tube during reconstitution.

Data in Table I show that as the concentration of cortisol in methylene chloride is increased, there is a small concomitant increase in the analytical recovery.

TABLE I

ANALYTICAL RECOVERY AT VARIOUS CONCENTRATIONS OF CORTISOL

An 8-ml volume of cortisol solution in methylene chloride was evaporated to dryness and reconstituted with 100 µl of mobile phase

Solution evaporated	*Analytical recovery of cortisol*		
	Experimental (µmol/l)	*Theoretical (µmol/l)*	*%*
0.04	1.4	3.2	44
0.12	5.1	9.6	53
0.40	20.6*	32.0	64
1.20	62.8**	96.0	65
4.00	246***	320	77
8.00	454 §	640	71

* Reconstituted sample (50 µl) diluted four-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

** Reconstituted sample (50 µl) diluted ten-fold with mobile phase and peak area compared to 9.6-µmol/l standard.

*** Reconstituted sample (50 µl) diluted 40-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

§ Reconstituted sample (50 µl) diluted 80-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

Chromatograms showed no extraneous peaks at 242 nm, following sample preparation, only a reduction in peak height compared to a cortisol standard prepared in our mobile phase. The relationship between recovery and concentration in Table I is inconsistent with a solubility problem associated with the reconstitution step, since concentrations exceeding the limit of solubility of cortisol would show reduced recoveries with increasing concentrations. Similarly, solubilities reported[12] for cortisol in water (0.77 mmol/l) and methanol (17 mmol/l) are well above the largest concentration examined in Table I. Unfortunately, the analytical recovery observed for the 0.04-µmol/l solution of cortisol in methylene chloride which showed the lowest value

TABLE II

ANALYTICAL RECOVERY OF CORTISOL FROM VARIOUS CONTAINERS

Type of tube	*Analytical recovery**	
	µmol/l	*%*
Pyrex glass	1.57	49
Low-actinic glass	1.94	61
Siliconized glass**	1.40	44
Polypropylene	0.98	31
Ethylenetetrafluoroethylene	1.71	53

* The theoretical concentration of a reconstituted specimen with 100% recovery is 3.20 µmol/l.

** Pyrex glass treated with Dow-Corning 360 fluid.

in Table I corresponds most closely to a typical physiological concentration in human serum before extraction with a ten-fold volume of solvent.

Table II shows that low analytical recovery of cortisol (*i.e.*, 31–61 %) was also observed in a variety of containers used while evaporating the methylene chloride prior to measurement by HPLC. Again no extraneous peaks were observed in the chromatogram at 242 nm. The analytical recovery of cortisol in a low actinic glass tube was only slightly higher than in a clear glass tube, suggesting the loss of steroid is not the result of photolysis to a non-absorbing compound during sample preparation. Assuming that a portion of the cortisol becomes irreversibly bound to the surface of the container during solvent evaporation, data in Table II suggests that the composition of the surface of the container has a negligible influence on the apparent binding. For example, coating the surface of the glass tube with dimethyl polysiloxane (*i.e.*, Dow-Corning 360 fluid) and the use of polypropylene and ethylenetetrafluoroethylene tubes failed to improve the analytical recovery compared to the untreated glass tube.

We further investigated the possible decomposition of cortisol during sample preparation by use of tritiated cortisol. Fig. 1 shows that the retention time of tritiated cortisol measured by radioactive counting in fractions collected during chromatography coincides with the retention time for cortisol measured by absorbance. There were no other peaks in the radiochromatogram and the calculated recovery of radioactivity in the eluted fractions was 71 %. These findings reaffirm the analytical loss of cortisol during sample preparation and suggest that the portion of cortisol

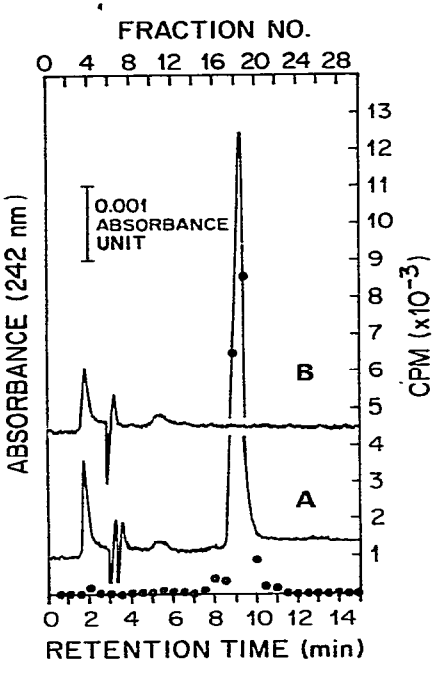

Fig. 1. Chromatogram (A, solid line) and a radiochromatogram (●) for cortisol and tritiated cortisol following sample preparation for HPLC. Chromatogram B shows a blank, consisting only of methylene chloride without cortisol, treated the same as the sample in A.

TABLE III

ANALYTICAL RECOVERY OF CORTISOL AT VARIOUS CONCENTRATIONS OF POLYETHYLENE GLYCOL

Solvent: methylene chloride. Cortisol concentration: 50 nmol/l.

Concentration of polyethylene glycol (mg/l)	*Analytical recovery**	
	μmol/l	*%*
0.000	2.16	54
0.004	2.27	57
0.01	1.70	42
0.10	2.34	58
0.40	3.48	87
1.00	3.62	90
2.00	3.67	92
5.00	3.85	96
10.0	3.96	99
20.0	3.95	99
50.0	3.96	99

* The theoretical concentration of a reconstituted specimen with 100% recovery is 4.00 μmol/l.

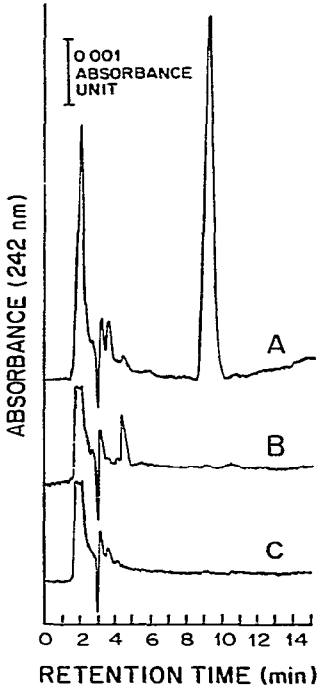

Fig. 2. Chromatogram A shows cortisol and polyethylene glycol following sample preparation for HPLC. Chromatograms B and C show blanks (no added cortisol) consisting of methylene chloride with and without polyethylene glycol prepared the same as the sample in A.

which redissolves in our mobile phase has not undergone chemical decomposition, *i.e.*, unless the product of such a decomposition has the same chromatographic retention time as cortisol and does not contain the radioactive portion of the molecule.

Collectively, the data presented above suggest that a portion of the cortisol becomes bound to the surface of the container during the evaporation step of sample preparation so that it is not readily redissolved in our mobile phase. We investigated the possibility of competing with the binding to the container by adding a material to the methylene chloride which would provide a matrix for cortisol in the residue produced during solvent evaporation. We selected polyethylene glycol as a candidate matrix material because it is soluble in methylene chloride, water and methanol and it is commonly used as a large molecular weight matrix component for lyophilized products. Table III shows that analytical recoveries of cortisol are increased with increasing concentrations of polyethylene glycol to a maximum of 99%, *i.e.*, even at a concentration of 1 mg/l of polyethylene glycol in methylene chloride the recovery increased to 90%. At a polyethylene glycol concentration of 10 mg/l and greater, values for the analytical recovery appeared to plateau at essentially the theoretical value.

Fig. 2 shows the effect of adding polyethylene glycol on the chromatogram for cortisol. Although several small extraneous peaks arising from polyethylene glycol are introduced in the beginning of the chromatogram, none of them coincide with the peak for cortisol.

As a final experiment, polyethylene glycol added to methylene chloride extracts prepared from an aqueous standard (500 nmol/l) gave analytical recoveries of cortisol ranging between 92 and 99% ($n = 7$, mean 96%). Although we have not examined different commercial sources of polyethylene glycol or the long-term effects of this polymer on our reversed-phase column, these results indicate that adding a suitable macromolecular matrix material to the methylene chloride before solvent evaporation substantially improves the analytical recovery of cortisol and lowers the variability of results.

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