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Quality control in the determination of cortisol in plasma/serum by using, on every sample, two different three-step separation methods including ultrafiltration, restricted-access high-performance liquid chromatography and reversed-phase high-performance liquid chromatography, and contrasting results to immunoassays

Hans W. Mueller*, Jürgen Eitel

Clinical Neurochemistry, Department of Neurosurgery, Justus-Liebig-University, 35392 Giessen, Germany

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Abstract

Tests of HPLC columns with restricted access, polymer covered alumina, polymer, and different ODS phases showed that base–acid compatible ODS columns gave the best peak shapes of cortisol, internal standard, as well as of plasma/serum (P/S) matrix components. Further trials with cortisol in P/S showed that three separation steps were essential in order to obtain chromatographic data which were superior to immunoassay data. Also, sufficient confidence in results required determination of each sample with two newly developed separation methods: (a) pre-separation with a restricted access column, concentration of the desired cut with a 20 mm base–acid compatible ODS column, and analysis with a 250 mm column filled with the same ODS; (b) pre-separation with an ultrafilter followed by the last two steps in (a). For detection UV was preferred over fluorescence. This twin multistep chromatography showed that immunoassays were very treacherous in that they produced a spectrum of results ranging from good to untenable without any warning whatever about functionality. The measurement of official controls, with reference values derived via gas chromatography–isotope dilution mass spectrometry, also demonstrated the superiority of the double HPLC method.

Keywords: Cortisol

1. Introduction

In the course of neuroendocrinological studies a large number of plasma or serum samples (P/S) were subjected to commercial immunoassays [radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)] in order to estimate levels of various hormones, including cortisol [hydrocortison (Cort)]. A critical look at the relationship between concentration and decomposition counts or absorbance of those kits' standards indicates that the word "estimate" is indeed justified. Simple tests, for instance the repetition of an assay or a dilution series, show that such estimations can be very

*Corresponding author.

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ambivalent, especially in the case of Cort. Cross-reactions or interferences with other substances cannot be detected in patient samples with one assay kit, but are impressively demonstrated by non-parallelisms in the results of external quality controls (compare Ref. [1]). Some of the literature [2–6] on HPLC of Cort seems to corroborate this negative portrayal of immunoassays. These HPLC methods, unfortunately, also appear to be somewhat equivocal in that they have only weak, if any, quality checks intrinsic to the unknown samples themselves. The initial thrust of the present effort was, then, to find out whether some published separations were so efficient as to justify the general neglect of possibly hidden interference in some P/S samples, if not in all. Normal-phase HPLC was not considered since multifunctionalities, especially those capable of hydrogen bonding, like Cort's hydroxy groups, often produce undue tailing. A single-step separation was believed to bear a chance at success only with restricted access columns (no reports found), as ODS and similar columns appear to operate with a fickle protein (or other partially retained material) stationary phase upon P/S injection [7]. Also tried were our own improvements of the most widespread two-step separations, the first step traditionally being a liquid–liquid extraction [4–6,8–11], as well as our own variations (early results with restricted access column: [12]) on three-step chromatography (general [13], Cort [14]) and three-step separations with an ultrafiltration as a first step [12,15,16]. The unsolved problem of undetected overlapping was addressed by determining all P/S samples via two different methods which, and this represented the greatest difficulty, had to be refined until they both functioned under clinic-like routine conditions and until the results agreed reasonably. This was done with pooled P/S and P/S from which immunoassays had been performed. Cats' P/S was also examined. Except for a short stint with fluorescence, UV detectors were used.

A digression on terminology is in order here. The authors prefer to limit "multidimensional" to planar chromatography (TLC). Multistep chromatography, or multistep separations if other purifications are involved, describe the consecutive methods more succinctly.

2. Experimental

2.1. Equipment

Instead of reciprocating piston pumps the nitrogen pressure vessel system JN 1 (BCMA from ERC, Altegolfsheim, Germany), modified for ease of handling and supplemented with 1/2-inch pipes (Autoclave Engineers, Erie, PA, USA) and needle valves (SSI, State College, PA, USA) for flow control, was used to push the mobile phase through the columns. Flow-rates were monitored with an air bubble flow meter of our own design. It consisted of a 200-μl graduated glass pipet (Brandt, Wertheim, Germany) on which the tapered tip was cut off in order to fasten specially manufactured Bola teflon fittings (Bohlender, Lauda, Germany). These allowed insertion of the pipet into the 1/16-inch capillary coming from the detector and connection of a 1-ml syringe (insulin) for placing an air bubble into the liquid path. Both 7125 and 8125 injection valves as well as 2094 and 7000 switching valves (all Rheodyne, Cotati, CA, USA) were employed. Though lesser detectors were used for some preliminary runs the numerical data of this report were obtained with the programmable UV detector SA 6503 (Severn, Shefford, UK, a Linear detector) at 250 nm; fluorescence detection was done with a F-2000 fluorescence spectrometer with a flow-through cell (Hitachi, Tokyo, Japan) at various excitation and emission wavelengths.

Integrations and concentration calculations were performed on a HP 3394 A integrator (Hewlett Packard, Avondale, CA, USA) using the internal standard method on peak area until it was noted that peak hight was more reliable. Data of both modes are included in this report.

2.2. Materials

Water was obtained from a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA) and methanol (Rathburn, Walkerburn, UK) was HPLC grade, while sodium sulfate (dry), potassium dihydrogenphosphate, sodium and lithium dodecylsulfate (SDS and LDS), dithiothreitol, and polyethyleneglycol (PEG)

were all analytical or biological grades (Merck, Darmstadt, Germany). Monochloroacetic acid, the standards cortisol, corticosterone, nortestosterone, fludrocortisone, and dexamethasone (all about 99% purity) were used as delivered (Sigma, St. Louis, MO, USA). All mobile phases were filtered through 0.45-μm regenerated cellulose microfilters of 47 mm diameter in a corresponding glass apparatus (Sartorius, Goeppingen, Germany). The ultrafilters, with a cut-off at 30 000 g/mol, were Centrifree and Microcon-30 (Amicon, Beverly, MA, USA). The nitrogen pressurizing gas was of 99.996% purity (Messer-Griessheim, Griessheim, Germany). Columns and filling materials were: 4.6 × 250 mm Hypersil ODS (Shandon, Cheshire, UK), 4 × 250 and 2 × 20 mm ODS-I (4 × 250 column: SGE, Ringwood, Australia; all SGE columns are glass-lined, here filled with Inertsil ODS, Gasukuro Kogyo, now GL Sciences, Tokyo, Japan; 2 × 20 column: Upchurch, Okabor, WA, USA, stainless steel, dry-filled with Inertsil ODS in this laboratory), 4.6 × 250 PEEK and 2 × 20 mm LiChrospher-RP-Select B (4.6 × 250 column: MZ, Mainz, Germany, ODS filling from Merck; 2 × 20 column: as above, respectively), 4.6 × 250 Pinkerton and 4.6 × 150 mm Pinkerton II ISRP (Regis, Morton Grove, IL, USA; ISRP is internal surface reversed phase, thus restricted access with tripeptide inner surface), 4.6 × 250 mm SPS–ODS (lend by ict-ASS, SPS is "semi-permeable surface", so also a restricted access type, here with internal ODS surface), 4.6 × 250 mm Hisep (Supelco, Bellefonte, PA, USA filled with a "shielded hydrophobic phase", also restricted access), 4 × 250 mm Unisphere-PBD Alumina (MZ, SGE column filled with polybutadiene covered alumina from Biotage, Charlottesville, VA, USA), 4 × 100 and 2 × 10 mm MP-1 (filling material from interaction, Mountain View, CA, USA, the 4 × 100 mm SGE column was filled by MZ, the 2 × 10 mm is an Upchurch PEEK column and was dry-filled in this laboratory). The columns are given a self-explanatory name hereafter.

Cortisol RIA (DPC, Los Angeles, CA, USA) and ELISA (elias, Freiburg, Germany) were used essentially as recommended by the manufacturer. The ELISA were classified into three performance quality groups according to closeness of double determinations, quality controls, etc. As will be seen, detailed criteria need not be mentioned.

External quality controls were Lyphocheck (Bio-Rad, Hercules, CA, USA) and Ringversuche (Zentrale Referenzinstitution, Bonn, Germany; Ringversuche are the official German external quality assessment scheme, their reference values are based on GC–isotope dilution MS or GC–IDMS).

2.3. Standard solutions and sample sources

Stock solutions of standards were prepared by weighing close to 25 mg of the steroid into a 25-ml volumetric flask and filling to the mark with 50 H_2O–50 MeOH (%, v/v). Calibration standard was then obtained by diluting, usually with the same solvent, 25 μl of such a cortisol (Cort) stock solution, as well as 25 μl corticosterone (Cortico) or dexamethasone (Dex, avoids interference by endogenous Cortico) stock solutions (internal standard in the calibration standard) to 25 ml. The internal standard to be added to samples consisted of 50 μl of Cortico or Dex stock solution diluted to 10 ml as before. All solutions were distributed into brown teflon-capped vials, and stored at −25°C. Changes were only observed if solvent evaporation was not prevented. This internal standard was then mixed in the proportion of 40 μl to 200 μl P/S. Preliminary experiments were sometimes performed with different steroid concentrations, also including internal standards nortestosterone or fludrocortisone in either water–methanol or pooled plasma (Pool). With 40 H_2O–60 MeOH mobile phase on the Inertsil fludrocortisone eluted just barely ahead of Cort and was not used further as the other standards came at positions with less interference. Nortestosterone was retained too strongly to be useful.

Except for some samples of normal persons, HPLC was carried out on leftover frozen P/S of patients with pituitary problems or with subarachnoidal bleeding. All selected samples had their Cort determined via ELISA, only eleven of the oldest samples (about five years old) also with RIA. P/S from cats was collected and frozen during experiments involving brain lesions due to local pressurizing. Lyphocheck and Ringversuch samples were all reconstituted and stored in a freezer at −26°C

until use, some for several years. HPLC of Lyphocheck samples was discontinued early, because of enormous interference in a few. Lyphochecks without this unknown material chromatographed very well.

2.4. Preliminary analytical trials

A brusque account of the trials which led to the final multistep separations is essential in the development of the arguments in this paper. The suitability of all columns was screened with the standard solutions and water–methanol mobile phases. A finer screening and the initial chromatographic development called for substituting Pool and Pool spiked with Cort and internal standard for the standard solutions. Occasionally, individual's P/S and individual's spiked P/S was also used. The tested columns are listed in Section 2.2. Mobile phase flow-rates were between 0.5 and 0.25 ml/min. Individual or initial columns were protected with a 2 × 20 mm column of the same filling as the main one. A step gradient was applied to some restricted access columns by substituting a mobile phase with higher MeOH content after front peaks had eluted. Protein modifications with MeOH, EtOH, Na_2SO_4, KH_2PO_4, PEG [17], SDS [13,18] and LDS were also tried on the restricted access columns. All reversed-phase columns had only ultrafiltrate (centrifree) of the biological samples injected.

Furthermore, the widespread technique using a liquid–liquid extraction as first step [4–6,8–11] was modified by substituting the base–acid compatible LiChrospher or Inertsil for columns used in the literature. Other attempts at improvements included the insertion of a 0.25 *M* NaOH wash [5,9] ahead of the acid (0.2 *M* H_2SO_4) wash after chloroform extraction, and a pre-extraction with hexane or pentane [10].

In another modification of the two-step method the Pinkerton column was substituted [12] for the chloroform extraction (recently, a similar use of an ISRP column in Cort HPLC was published [19]). A fraction including the Cort and Cortico peaks (not visible) was collected (up to 3 ml) and completely injected on the Inertsil.

Lastly, this system was used to test the feasibility of a new three-step separation by inserting the 2 × 20 mm Inertsil between the Pinkerton and Inertsil (Fig. 1). The 2 × 20 mm Inertsil was selected after

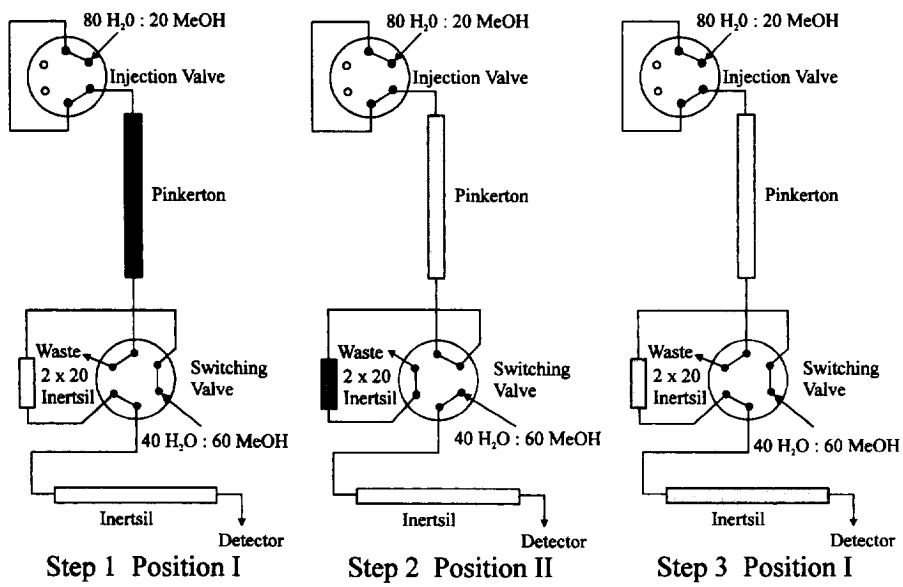

Fig. 1. The three steps of the Pink method shown schematically. The shading of the columns denotes the separation process. For the U-fil method the Pinkerton is removed and the first step replaced by the ultrafiltration, i.e. only steps 2 and 3 of the scheme without the Pinkerton apply.

testing it along with the 2 × 20 mm MP-1 and the 2 × 20 mm LiChrospher with calibration standard and Pinkerton mobile phase (80:20 H_2O–MEOH), later stepping up to Inertsil mobile phase (40:60 H_2O–MeOH). A twin to the three-step method was found in substituting ultrafilters for the Pinkerton. Pool, spiked Pool, or the individual's P/S counterpart was used to adjust the systems to about 100% recovery. The later developments are described in detail below.

Combinations of the literature methods employing alcoholic H_2SO_4 [9,11] (ref. [9] is fluorometry) were applied using only the Inertsil.

2.5. Final analytical developments: the twin three-step methods

(a) The three-step method using the Pinkerton as initial step (Pink method, Fig. 1)

Step 1. For 9 min after the injection of 20 μl calibration standard or 50 μl P/S (containing 40 μl sample internal standard per 200-μl sample) the Pinkerton effluent (80:20 H_2O–MeOH) was passed into waste at a flow-rate of around 0.5 ml/min. Later it was adopted to add about 100 mg of solid Na_2SO_4 to the 200 P/S + 40 standard, separating solids by centrifugation, and injecting 75 μl.

Step 2. The switching valve was toggled (Fig. 1, position II) so that the Pinkerton eluted onto the 2 × 20 mm Inertsil and from there into waste at a flow-rate of about 0.36 ml/min. This position was held for 30 min to collect all of the Cort and internal standard (most often Dex).

Step 3. The switching valve was returned to the initial position (I) such that the 40:60 H_2O–MeOH transported the Cort and internal standard onto the Inertsil. The flow rate of the 2 × 20 mm Inertsil and Inertsil was kept near 0.33 ml/min. In most cases the switching valve was again repositioned after 1.5 min to connect the 40:60 H_2O–MeOH only to the Inertsil. The flow-rate was then about 0.35 ml/min. The mobile phase was allowed to continue flowing on the Pinkerton and 2 × 20 mm Inertsil during this time.

(b) The three-step method using the ultrafilters in the initial step (U-fil method)

Instead of the Pinkerton an ultrafiltration initiated the separation here. Pretreatment of P/S was also checked, including addition of monochloroacetic acid [15], and various amounts (solids up to saturation) of PEG, Na_2SO_4, SDS, EtOH, MeOH. The final sequence was initiated after adding, in a microvial, 200 μl MeOH to the 200 μl P/S + 40 μl internal standard mixture, centrifuging (microcentrifuge), and placing the supernatant on the ultrafilter:

Step 1. The above supernatant in the centrifree ultrafilter was placed in a cooling centrifuge and spun at 2000 *g* at 4°C for 30 min to 2 h, depending on P/S. When the microcon filters became available they were found to be convenient replacements, giving identical results, even though 2–4 h were required in microcentrifuges at room temperature. The ultrafiltrations were usually terminated before completion.

Step 2. A 100-μl aliquot of the ultrafiltrate was injected on the 2 × 20 mm Inertsil (Fig. 1, step 2, position II, without Pinkerton), running on 80:20 H_2O–MeOH. The emanating mobile phase was allowed to go to waste for 20 min at a flow-rate of about 0.4 ml/min.

Step 3. The switching valve was toggled (Fig. 1, step 3, position I, no Pinkerton) to let 40:60 H_2O–MeOH flow through the 2 × 20 mm Inertsil, washing remaining material onto the Inertsil, flow-rates were kept close to the corresponding ones in the Pink method. Also as above, the valve was again reversed after 1.5 min in order to isolate the Inertsil chromatography. The 2 × 20 mm column was sometimes washed with methanol or 2-propanol during this time, equilibrating the column for about 5 min with mobile phase before the next injection.

In both the Pink and U-fil methods injection volume was normally determined by the loop, not the syringe. Spectra were taken occasionally at 200–300 nm in the stop flow mode (here possible by placing the injection valve between load and inject and/or by closing a needle valve) on some unknowns as well as on high and low baselines.

(c) Linearity of the three-step methods

Either 200 μl water or Pool plus, in each case, a 40 μl Cort sample internal standard solution were partially and sequentially diluted with water or water–methanol, respectively Pool, to 1:64 or 1:128. To 200-μl aliquots of all of these were added 40 μl of the sample internal standard solution (Cortico,

later Dex). Up to two higher Cort concentration samples were prepared by addition of 80 or 120 μl of the mentioned Cort solution to 200 μl water or Pool and again adding 40 μl sample internal standard to 200-μl portions. The determined Cort concentrations were then compared with calculated values using least-squares linear regression of Excel (university licence from Microsoft, Cambridge, MA, USA).

The linearity could also be tested with real patient samples under routine conditions, because lower injections than normal could not always be avoided. Here the variation of chromatographed amounts occurred in the internal standard as well as in Cort.

(d) Recovery of the three-step methods

One type of recovery was ascertained by comparing Pink or U-fil method peak heights of samples with peak heights of calibration standards or to mixtures of 200 μl water plus 40 μl sample internal standard, both injected directly on the Inertsil. This technique represents an approach to absolute recovery.

A relative recovery, that is, the comparisons of Cort concentrations of the calibration standards (at least one a day or every time something was modified, like changing a frit) were used to monitor performance of the two methods. A greater than 5% drift was supposed to initiate remedies. Peak heights were also used in this manner, but, in the case of P/S, only semi-quantitatively. Methanol evaporation and composition changes in the U-fil and sodium sulfate addition in the Pink method (undefined volume) prevented more. The linearity results also contain information on relative recovery.

(e) Sensitivity of the three-step methods

The sensitivity was cleaned from linearity experiments, from data which resulted from lower than normal volume injections, and from samples with low Cort concentrations.

2.6. Column maintenance

When the performance of the large columns abated they were individually tested with calibration standard and, if required, treated with an aqueous solution of 3%, by weight, LDS (has much better solubility properties than SDS) in 0.025 *M* dithiothreitol [18] at a flow-rate of 0.5 ml/min (if possible) for at least 1 h or until the flow-rate no longer increased. After washing with water the column was again tested with a calibration standard (Example: Fig. 2b). When the 2 × 20 mm Inertsil was suspected it was simply refilled. The Inertsil almost never required such harsh cleaning.

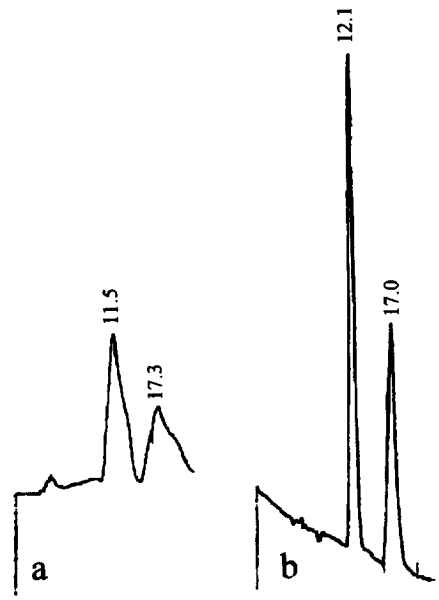

Fig. 2. Chromatograms of calibration standard obtained with the Pinkerton directly connected to the detector. The peaks with retention times are Cort and Dex in order of increasing time. (a) Column deteriorated by P/S samples, and (b) after cleaning with a LDS–dithiothreitol solution.

2.7. Calculations

Linear regression (95% confidence level), means of Cort concentration differences, the corresponding normal S.D. (N.S.D.), and the arithmetic were elucidated with Microsoft Excel. The N.S.D. (*n*, the number of measurements instead of $n-1$ in the denominator) was used as the data are treated as a complete unit.

3. Results

3.1. Preliminary analytical trials

The MP-1 had to be eliminated on the basis of its unfavorably high retention and extreme tailing. The restricted access columns did not separate matrix components of the biological fluid, except in a few samples, which eliminated the one-step approach. Protein modification did not help, especially PEG, SDS and LDS caused additional problems, so that it was not surprising to learn later that detergents can also be problematical when added to the mobile phase [13]. That proteins were not the main problem was shown by the mobile phase step gradient: the second solvent applied after the proteins had eluted produced peaks rivaling the front peaks in every negative respect. The ultrafiltration used with the reversed-phase columns eliminated most of the Cort and internal standard along with most of the matrix, so that this two-step method was only useful to find the most advantageous of the remaining reverse-phase columns. The Hypersil, here considered to be a representative of the columns used in published reports, presented with relatively unfavorable tailing especially of the matrix, while the Alumina was even worse in both respects (slightly lower retention than the Hypersil). The analytes, but even more so the matrix components, emerged much sharper from the LiChrospher and Inertsil (both higher retention than the Hypersil). The latter column gave strikingly better resolution in a few samples, making it the column of choice.

It was then not surprising that the use of the Inertsil in trials with the liquid–liquid extraction method allowed a resolution and sensitivity improvement over comparable reports in the literature. A pre-extraction with hexane or pentane was without effect. The insertion of the NaOH washing step decreased unknowns, but not enough. Matrix components still created one sharp peak next to or overlapping with another. Since their heights were on the order of the Cort values (some even overtowered the spike) the precision was far too low.

When the Pinkerton was used for pre-separation of Pool or P/S it was immediately apparent that this opened a path to follow. The Pinkerton II and SPS could not be used, because of their higher analyte affinity (retention approaches Hypersil's). This would have required a mobile phase step gradient the second solvent of which would have caused a wash-in on the Inertsil. The Hisep (retention similar to Pinkerton) could not be used for clinical applications. All suggestions by the vendor, including continuous flushing with mobile phase for 48 h and injecting sample without stopping flow, failed to stop bleeding. This two-step method was discontinued when it was realized that some patient samples required a third separation step.

The 2 × 20 mm columns, used as the intermediate in the three-step methods, had the following affinity for the steroids in decreasing order: MP-1 >> Inertsil > LiChrospher. Only the Inertsil had the right affinity to function as a concentrator yet release the analytes fast enough to guarantee sharp peaks on the Inertsil.

Conventional solid-phase extraction [20] was not examined, since even the 250-mm columns are short of sufficient.

Only broad inhomogeneous peaks resulted when H_2SO_4 was used (pre-column) to produce fluorescence. The injections of high concentrations of the acid were deleterious to the column. The experiments were terminated before a newer report [21] appeared in which H_2SO_4 was removed before injection. High predominance of one product was claimed. This did not rekindle appetite for more, because it is felt that adopting the technique, if it really holds its promise, to clinical routine should be difficult. Furthermore, the sensitivity advantage of fluorescence dwindles when the criteria of this paper are applied. Also, Dex fluoresced only meekly.

3.2. Final analytical developments: the twin three-step methods

(a) The Pink method

The small intermediate column not only concentrated the eluent from the Pinkerton but also removed more unknowns relative to the corresponding two-step method. Now the whole sequence required only a simple switching act, a great help in the clinical environment. Fig. 3 shows typical chromatograms. As confidence grew there was continuously less

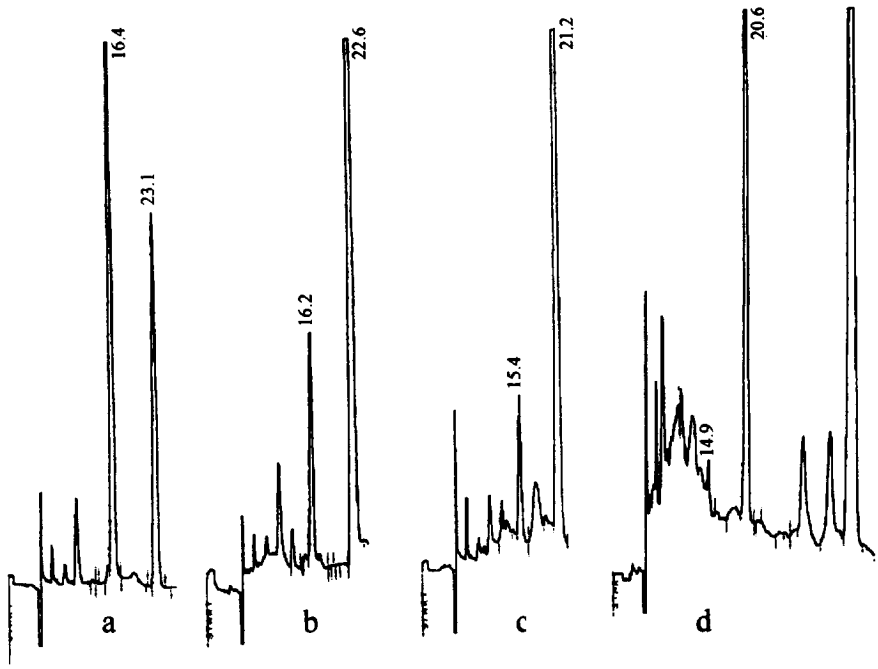

Fig. 3. Representative chromatograms of the twin three-step methods. The peaks with retention times are Cort and Dex in order of increasing time. Chromatograms are of (a) calibration standard obtained with the Pink method, (b) a relatively clean patient sample also with the Pink method, (c) another clean patient sample with the U-fil method, (d) a patient sample (14d in Fig. 4) among the most difficult ones encountered with the U-fil method; integration had to be performed manually. Cort concentrations were: (a) 102.6 μg/100 ml (should be 100.2), (b) 16.3, (c) 9.2, (d) 1.9. Large sharp unknowns like the one after Dex in (d) were rare.

sample discrimination (for instance, a relatively trouble-free Pool was no longer used). Promptly, flow problems increased primarily in the Pinkerton. P/S from some patients is prone to cause severe restriction or even immediate complete plugging of column frits. This led to the reintroduction of sodium sulfate for protein precipitation. This modification is not counted as another step since all samples were spun down at some point. Flow variations, which rendered the chromatography useless, seldom occurred hereafter (this is another reason for lower discrimination: fewer chromatogram rejections). Cort is not reduced by this partial protein precipitation. This procedure led to splitting some data interpretation into groups of before and after Na_2SO_4. As work became more routine the heart cut (step II in Fig. 1) needed extension to 30 min, because of Pinkerton phase changes (Fig. 2a) and remaining smaller retention time variabilities. All in all, the adoption to clinic-like operation brought fewer failures, but a higher rate of difficult chromatograms. Though these broader peaks did not interfere with integration as severely as the relatively much larger and partially sharper unknowns (even in low problem samples!) of the preliminary techniques they did, unfortunately, prevent a more pronounced improvement in data correlation.

(b) The U-fil method

The direct ultrafiltration of P/S was shown in this context to give no more than 20% recovery. When the same concentration of monochloroacetic acid as in the literature [15] was used a maximum of 25% recovery obtained. Ethanol proved useless, because it closed the filter pores. SDS and especially PEG also presented with similarly low recovery. The methanol technique finally adopted seems to function not only via denaturing of proteins, but also by increasing solubility of Cort as was also observed in solid-phase extraction [20] and in "direct injection" HPLC [13].

In analogy to the Pink method an ever increasing number of samples caused problems, here plugging of the filters. For this reason the samples were first centrifuged and only the supernatants placed on the ultrafilters. Again the centrifugation is not counted as an extra step. Another parallelism to the Pink method occurred in that here also were failures reduced, but difficult chromatograms appeared more often as sample discrimination was lowered.

(c) Comparisons of Pink, U-fil, ELISA, and Ringversuch methods

The amount of data allows only a representative presentation of Cort concentrations, namely those of the Ringversuche (Table 1). Instead, the data (including those of Table 1) were condensed to actual and absolute average (mean) concentration differences and corresponding mean percentage differences and presented in Table 2. By comparing actual and absolute mean differences one sees immediately that immunoassays usually yield higher Cort concentrations than do either the present HPLC or the Ringversuche (GC–IDMS). The positive and negative variations between the latter two are nearly balanced. The larger N.S.D. of the actual mean differences pay tribute to the fact that deviations are not only unidirectional. The most obvious result is the relatively huge spread of the immunoassay data, and that patients' P/S yielded the worst data by far (range of absolute differences between ELISA and total HPLC: 0–543%, the range between Pink and U-fil: 0–80%). The ELISA data deviations did not

Table 1
Determination of Cort in Ringversuche with Pink, U-fil, and ELISA

Sample	Pink a	Pink b	U-fil a	U-fil b	Av HPLC (H)	ELISA (E)	Ref (R)	R Range
91 1A	29.2		26.0	25.2	26.8	35.9	26.45	17.72–35.18
91 1B	33.1	34.0	30	30.7	32	42.3	31.5	21.01–42.03
91 2A	8		7.0	8.3	7.8	2.2	6.85	4.57–9.13
91 2B	14.5				14.5	6.2	16.12	10.80–21.45
91 3A	10		10.3	12	10.8	6.2	10.69	7.14–14.2
91 3B	20.3		18	19.5	19.3	15.3	20.11	13.44–26.78
91 4A	16.9	16.7	16.3		16.6	15.4	16.12	10.80–21.45
91 4B	33.2	34.5	28.7	29.3	31.4	28.2	31.45	21.05–42.03
92 1A	7.7	7.5	7.8	8.8	7.9		6.85	4.42–9.28
92 1B	11.4	11.9	9		10.8		10.69	8.01–13.37
92 2A	18.8	19.0	17.4	17.4	18.2	12	20.11	14.67–25.54
92 2B	25.5	26.5	21.6		24.5	18.8	26.45	19.28–33.6
92 3A	31.7		32.2		32	26.3	31.45	22.93–40.22
92 3B	7.9		7.1		7.5	19.1	6.85	4.42–9.28
93 1A	16.7		14.7		15.7	26.3	14.1	10.5–17.6
93 1B	6.7		5.5		6.1	19.2	5.5	3.6–7.5
93 2A	4.4	4.4	5.9		4.9	8.5	5.5	3.6–7.5
93 2B	13.4		13.6		13.5	15	14.1	10.5–17.6
93 3A	27.6		27.8	27.3	27.6	33.2	25.7	19.6–34.2
93 3B			33.2	34.4	33.8	37.7	29.3	24.7–43.1
93 4A	5.8		7.9		6.9	8.7	5.3	3.45–7.2
93 4B	23.5		28.8		26.2	31.3	20.5	14.9–26.1
94 1A	14.5		16.1		15.3	14.2	14.1	10.4–17.7
94 1B	5.7		5.1		5.4	8.1	5.5	4.1–7
94 2A	33.6		33.5		33.6	36.2	33.8	24.7–42.9
94 2B	22.5		21.1		21.8	19	20.7	15.1–26.3
94 3A	8.6		6.7		7.7	10.2	6.99	5.14–8.84
94 3B	28.2		25.6		26.9	32.5	26.9	19.6–34.2
94 4A			20.3		20.3	20	20.7	15.1–26.3
94 4B	32.8		34.6		33.7	33.1	33.8	24.7–42.9

Cort concentrations are in μg/100 ml. Av HPLC is the average of the HPLC results. The Ref or R columns contain the Ringversuch reference values. a and b are separate intramethod determinations.

Table 2
Means ± N.S.D. of the differences between Pink, U-fil, ELISA, and Ringversuch methods in μg/100 ml as well as in percent

Sample	H−R	|H−R|	H−R/R %	|H−R/R|%	E−R	|E−R|	E−R/R %	|E−R/R|	*n*
Ring	0.5±1.5	1.1±1.2	4±10	7±7	1.9±6.6	5.6±4.0	23±65	45±53	30
	P−U	|P−U|	P−U/H %	|P−U/H|%	H−E	|H−E|	H−E/H %	|H−E/H|%	
Human a	0.5±2	1.6±1.4	4±16	14±12	−0.7±9.0	6.0±6.7	−11±71	45±57	129
Human b	−0.2±1.8	1.4±1.2	−2±20	13±15	−11.3±6.6	11.6±6.2	−94±87	95±88	43
Cat	−0.1±1.0	0.8±0.7	0±7	6±4					9
RIA			−27±18		−3.0±5.9	4.2±4.8	−30±66	42±63	11

H−R/R % stands for (Σ 100 (H−R)/R)/n ± N.S.D., etc.; the capital letters are Cort concentrations: H, average HPLC = (P+U)/2; R, Ringversuch; P, Pink; U, U-fil; E, ELISA or RIA. P and U are averages if more than one intra-method determination exists. Means derived from absolute differences are headed by symbols in | |. Human a are values obtained before use of Na_2SO_4 in the Pink method. Human b are those obtained after Na_2SO_4.

correlate with the performance quality of the individual ELISA. The −27% for HPLC of the "RIA samples" are a chance result, a correlation of data differences to storage time does not exist. Also, there is no discernible dependency on whether a few hours or as much as 2 years elapsed between parallel measurements. Inter-HPLC comparisons are practically no worse than the intra counterpart (Table 1; for brevity's sake 55 other intra-method values are not shown), indicating that differences are not due to hidden single peak overlapping and that both methods are at a similar state of reliability. Of the 76 differences between the HPLC methods which are above 10% there are 31 (out of 56) due to samples with 10 μg/100 ml or less. The differences are also less pronounced in Table 1 than in patient data, and that, even though many Ringversuch samples presented with huge unknowns reminiscent of Lyphocheck samples. Yet only Ringversuch 94 4A (Pink) could not be integrated; in some others huge unknowns were treated as baselines by the integrator. Cat's P/S is equally amenable to Pink and U-fil methods as are the human fluids.

All chromatogram pairs showing concentration differences greater than 10% (41% of all samples; greater than 15% differences were seen in 20% of all samples; greater than 20% differences were seen in 15% of all samples) were carefully re-examined in an attempt to elucidate the cause of the discrepancies. In one or two cases a single sharp peak under analyte in only one of the methods may have been responsible, all others were certainly the result of undulating baselines with characteristic method-dependent patterns. Since this background was also dependent on sample, and since calibration standards were much cleaner, accumulation and slow release of unknowns cannot be the major cause of the twin method differences. The culprit must be matrix material that even the Inertsil cannot chromatograph properly. The spectra of such matrix materials were quite broad but most peaked at 280 nm, some at 230 nm or at both. Obviously, the materials were not homogeneous. Aromatic groups or conjugated double bonds could be involved. Smoother baselines also gave such spectra, if less intensely, showing that some unknown accumulation does occur.

(d) Linearity of the three-step methods

Above 1:64 dilution all linearity tests gave excellent results. Data are given in Table 3. Curving-up of Cort concentrations at the lower dilutions was later eliminated by removing carry-over from the syringe and injection valve. Native Cort of Pool was subtracted from the spike before applying the linear regression. Carry-over was known in the series "H_2O 92 Pink" and subtracted. The correlation coefficient proved absolutely insensitive to such interferences which were severe in some of the lowest dilutions. To underscore the point the Cort concentrations in μg/100 ml are presented here for the case with worst and least carry-over, together with the calculated values shown in Table 4.

Fig. 4 represents linearity data cleaned from samples determined under routine conditions. It is clear that linearity is given.

(e) Recovery of the three-step methods

Comparisons of peak heights to those of occasional injections directly on the Inertsil gave the following "absolute" recoveries:

(1). For Cortico in Pool determined by Pink method compared with a corresponding solution in

Table 3
Linearity (dilution) data of the twin methods

Sample	Slope (S.E.)	Intercept (S.E.)	S.E.	Correlation coefficient	*n*
H_2O 94 Pink	1.006 (0.006)	−1.510 (0.485)	1.2074	0.9999	10
H_2O 94 U-fil	0.994 (0.011)	−0.400 (0.873)	2.1700	0.9995	10
H_2O 92 Pink	0.922 (0.003)	−0.149 (0.281)	0.7001	0.9999	10
Pool 91 Pink	0.906 (0.031)	2.507 (2.527)	5.6632	0.9965	8
Pool 95 U-fil	1.073 (0.023)	−1.079 (1.392)	3.1787	0.9984	9

S.E., standard error. Sample column: H_2O refers to standards in aqueous solution, Pool refers to spiked biological mixtures, the numbers refer to the year of the experiment.

Table 4
Examples of individual concentrations underlying Table 3

Sample	Concentrations (mg/100 ml)									
H_2O 94 U-fil	2.3	1.7	2.4	5.2	10.2	20.3	37.5	82.5	138.6	189.2
Calculated	0.7	1.3	2.6	5.2	10.4	20.9	41.8	83.5	142.9	187.5
Pool 95 U-fil	0.5	1.3	2.1	8.4	15.2	30	61.7	104.8	146.2	
Calculated	0.5	1.0	1.9	7.7	15.4	30.9	61.7	102.8	132.2.	

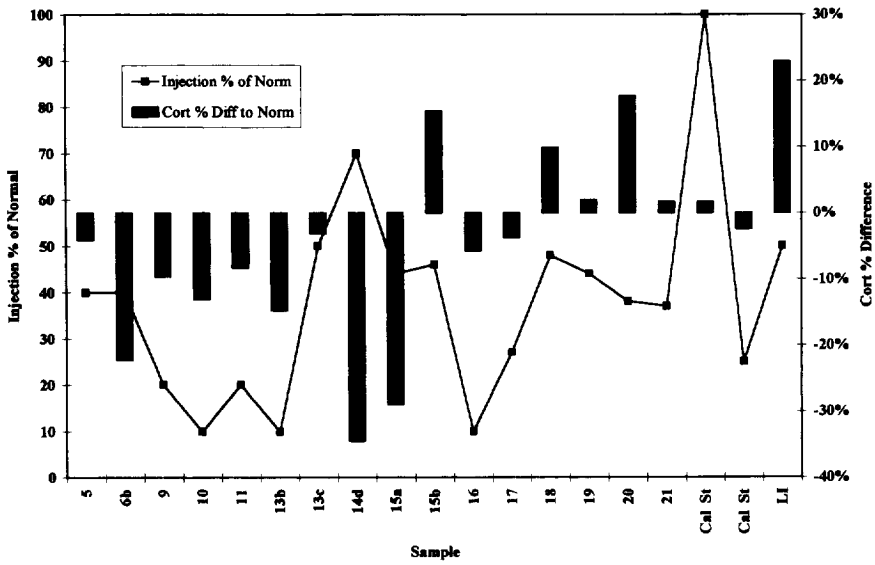

Fig. 4. Examples of Cort concentration differences in percent (Cort % Diff to Norm) between normal and subnormal volume injections. Cal St means calibration standard, LI is a Lyphocheck I sample. Injection % of Norm refers to the injection ratio: 100 × (subnormal volume/normal volume).

water (200+40) on the Inertsil alone: 97% (n = 9, data collected in one session).

(2). For Dex in Pool, Pink method, compared with calibration standard directly injected on Inertsil: 104% (n = 6, collected over 2 years).

(3). For the same except with U-fil method: 116% (n = 5, collected over 2 years).

(4). For four samples of Dex in water, determined with Pink or U-fil methods, compared with a similar solution directly injected on the Inertsil on three different occasions: 100% (collected over 2 months).

(f) Sensitivity of the three-step methods

A noise to signal ratio (S/N) of 4 was considered practical. At this S/N the limit lies at 1 μg/100 ml which corresponds to 0.5 ng/injection, the basis being a 50-μl injection of the normal 200 μl P/S plus 40 μl internal standard.

3.3. Column maintenance

Fig. 2a shows a typical chromatogram of a deteriorated Pinkerton, Fig. 2b presents a chromatogram of the reconstituted column looking as it should. After Na_2S0_4 was used to initiate the Pink method this extreme deterioration, which was noted by a reduction of the Dex peak in the Pink method, was not observed even though over 100 injections were involved. Without the partial protein precipitation the column decline occurred with 1–30 patient sample injections. Parallel to this Na_2SO_4 use a more rigorous regimen of washing all columns with methanol or iso-propanol at the end of a working day

and replacing the alcohol with a mobile phase overnight must have contributed to the above success. The removal of pure alcohol after washing definitely reduced the jump in flow resistance generally observed when alcohol was left on the column overnight.

4. Discussion

In chemistry [22,23] a single chromatographic method is not considered sufficient for identification or unequivocal quantitative determination of a compound. This study clearly shows again why this is a wise rule. One piece of evidence for this statement comes from the results of the preliminary trials (tests of the columns with ultrafiltrates) which showed the Hypersil to be inferior to the Inertsil. Since the Hypersil can be considered as representative of the ODS columns used in the more common HPLC of Cort [4,5], and since the improved liquid–liquid extraction method with the Inertsil proved useless, it follows that single column techniques need not be considered for clinical applications. In this setting it appears strange that the more carefully controlled HPLC analysis of Cort [24] known to the authors, though also not intrinsic to all samples, was applied to blood from cows. Now, it is highly interesting that at least one of the early practitioners of simple HPLC [25] also proceeded toward a three-step approach [14]. Obviously, the common methods of validation practices have led to some improvements over the years. They have not removed the uncertainty about failures with samples derived from real clinical processes. This ambivalence in chromatographic results can only be removed by the parallel application of two different techniques as is shown here. The presented data, having required painstaking development, clearly indicate that quality control extrinsic to the unknown analytes represents chance results. The terminology hinted upon must be elaborated. Quality control with all unknown samples themselves may be called intrinsic, while internal or external controls and spiking methods, which do not involve the unknown analytes, could then be called extrinsic. Traditionally, external refers to the like of Lyphocheck or Ringversuche, internal to own controls. Spiking requires a special comment. All methods described here were developed using spiking, so that the linearity experiments also represent spiking data. This, the continuous checks of absolute peak height in calibration and sample internal standards, and occasional injections of H_2O instead of sample obviate the use of further spiking.

The question remains as to what differences and quality of twin methods is required to qualify them for intrinsic validation. The difference between the Pinkerton and the ultrafilter of the twin methods imparts great confidence that interfering substances are differentially separated. Certainly, using immunoassays to validate HPLC [8,16,25] is no longer tenable. Immunoassays simply fail with some patients, irrespective of internal or external quality control results (performance class). Detecting at two or more wavelengths does not help as the baselines always have broad absorptions usually with the mentioned maxima. For this reason this technique, as well as taking spectra, is even disqualified as an intrinsic control; they are only useful in development. Though the above question cannot be answered precisely, a practical recommendation is possible. One needs to test different columns or other separation methods on relevant samples and then use the pair with the largest difference in the matrix background. Afterwards, the separation qualities must be developed until the desired precision is obtained.

The last determinations made prior to writing indicate the tendency that precision of the Pink/U-fil method is settling at a maximum difference of 10% if carry-over is eliminated, contamination of standards and solvents rigorously monitored and removed, integration performed with a personal computer system, and at least the present state of technicians' skill and knowledge kept up. To reduce differences considerably below 10% does not appear possible in three steps with available columns. Using four separation steps is an arduous possibility for further improvement. Full automation of the Pink and partial automation of the U-fil method (from step 2) are being sought and promise another slight upgrade. The ultrafiltration itself does not require much personnel time, while full automation [15] is expensive and might not work with the membranes used here. It may be noted that HPLC, or chromatography in general, if teamed with a highly specific

analytical tool like MS (the Ringversuch GC–IDMS or other chromatography–MS [26,27]) has a built-in intrinsic quality control, but only if each MS is examined carefully, i.e. MS is not without problems [27].

The arguments of quality, time, and expense are always pitted against each other when decisions are made about using ELISA or HPLC. In view of the results here there is no question that time must be secondary at present. Furthermore, a twin double determination as described here is hardly more time-consuming than the single method counterpart. Whether HPLC should be preferred over immunoassays in the clinic can no longer be the question [5], but rather whether Cort should be determined or not.

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