GLC Analysis of Hydrocortisone, Triamcinolone Acetonide, and Desonide in Culture Media of Mouse and Human Dermal Fibroblasts Using Flame-Ionization Detection

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Abstract □ A quantitative GLC assay with flame-ionization detection capable of detecting nanogram quantities of hydrocortisone, triamcinolone acetonide, and desonide in biological fluids was developed. This assay consisted of two extractions of the glucocorticoids from 1 *N* sodium chloride-treated cell culture media into ethyl acetate and subsequent double derivatization with methoxyamine and *N*-trimethylsilylimidazole. The chemical structures of methoxime–trimethylsilyl derivatives were confirmed by GLC–mass spectrometry. The methoxime–trimethylsilyl derivatives were stable for 24 hr. The applicability of this assay was demonstrated by studies of the glucocorticoid levels in L-929 and human dermal fibroblasts cell culture media over prolonged incubation (0–96 hr).

Keyphrases □ Glucocorticoids—GLC–mass spectrometric analysis of hydrocortisone, triamcinolone acetonide, and desonide in cell culture media □ GLC–mass spectrometry—analysis of hydrocortisone, triamcinolone acetonide, and desonide in cell culture media □ Hydrocortisone—GLC–mass spectrometric analysis, cell culture media □ Triamcinolone acetonide—GLC–mass spectrometric analysis, cell culture media □ Desonide—GLC–mass spectrometric analysis, cell culture media

Synthetic glucocorticoids vary in their clinical potencies as well as in their potential for causing dermal atrophy, a major adverse effect of long-term topical glucocorticoid therapy (1). Studies of the pathogenesis of this adverse effect have involved investigations of glucocorticoid effects on cell proliferation, protein synthesis, collagen synthesis, secretion of acid mucopolysaccharides, and other pleiotypic responses such as polyribosome formation and DNA synthesis (2, 3).

BACKGROUND

Cultured dermal cells have been used previously as models, and the incubation of glucocorticoids with cultured cells lasted from hours to days. However, no reports of assays of glucocorticoids in cell culture media appear to have been published. It was assumed that the amounts of glucocorticoids dissolved represented their biologically active concentrations and that the metabolism of glucocorticoids during incubation was negligible. Therefore, development of a GLC assay should help to clarify the validity of such assumptions. Furthermore, with a quantitative GLC analysis of glucocorticoids in cultured cell systems, the relevant biochemical data of glucocorticoid effects can be more precisely interpreted, especially in terms of dose dependence.

The requirements for the estimation of glucocorticoids in biological systems are that sensitivity should be in the low nanogram range and that the technique should identify possible metabolites or decomposition products. Due to ready instrument availability, GLC using flame-ionization detection was selected for the determination of glucocorticoids in cell culture systems.

Few quantitative high-performance liquid chromatography (HPLC) (4–9) and GLC (10–12) assays have been reported for the analysis of synthetic glucocorticoids in biological fluids. One HPLC assay (4) achieved sensitivity as low as 0.05 ng of budesonide/ml of plasma in dogs with the aid of radioactive detection. Amounts as low as 2–4 ng of triamcinolone acetonide, triamcinolone, and prednisolone were detected in rat muscle by GLC using electron-capture detection and trimethylsilyl derivatization (12).

Martin and Amos (10) reported a GLC–mass spectrometric assay enabling the measurement of plasma concentrations of prednisone and prednisolone as methoxime–trimethylsilyl derivatives to nanogram per milliliter plasma levels. So far, methoxime–trimethylsilyl derivatization has not been applied to the assay of synthetic glucocorticoids except for prednisone and prednisolone (10), although it was used successfully for the assays of naturally occurring glucocorticoids (13). No specific assay techniques have been reported for desonide.

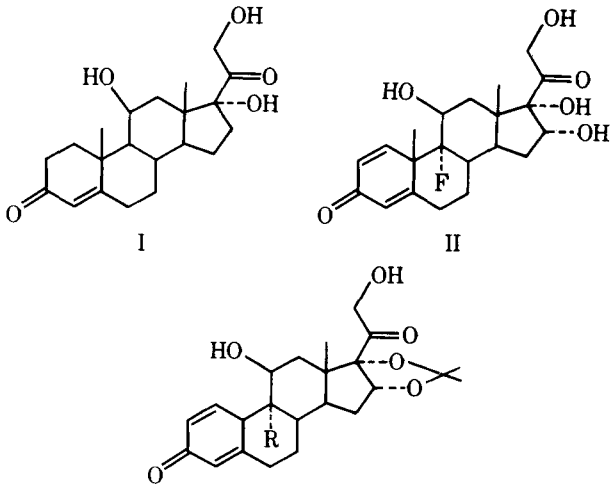
A GLC assay with flame-ionization detection was developed to quantitate hydrocortisone and synthetic glucocorticoids in cell culture media, using methoxime–trimethylsilyl derivatization.

EXPERIMENTAL

Materials—Hydrocortisone[1] (I), triamcinolone[2] (II), triamcinolone acetonide[2] (III), and desonide[3] (IV) were obtained commercially. Progesterone[4] (V) was used as the internal standard. Methanol[5] and ethyl acetate[5] were distilled in glass, and pyridine[6] was silylating grade.

Purity Confirmation of I–V—All steroids were dried at 40° for 1 hr and at ambient temperature overnight in a vacuum oven before use. The weight lost due to the drying was <1% in all cases.

The melting points of these compounds were determined by both capillary melting-point determination[7] and differential scanning calorimetry[8]. The purities of I, IV, and V were satisfactory as received.



[1] Lot 402-9511, Pfizer Co., Montreal, Canada.
[2] Compound II was USP, code 2014, batch Z5040, and III was USP, code 45645, batch 431, Cyanamid of Canada Ltd., Montreal, Canada.
[3] Lot CS-1-37, Dome Laboratories, Division of Miles Laboratories, West Haven, Conn.
[4] Lot 113C-0190, Sigma Chemical Co., St. Louis, Mo.
[5] Caledon, Georgetown, Ontario, Canada.
[6] Pierce Chemical Co., Rockford, Ill.
[7] Thomas-Hoover apparatus, Arthur H. Thomas Co., Philadelphia, Pa.
[8] Model 1B, Perkin-Elmer, Norwalk, Conn.

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Table I—Retention Time of Parent and Derivatized Glucocorticoids

Glucocorticoid	Retention Time[a], min: Underivatized Compound[b], 3% OV-17	Methoxime–Trimethylsilyl Derivative, 3% OV-17	Methoxime–Trimethylsilyl Derivative, 3% OV-7
Progesterone	1.31	1.38	2.57
Prednisolone	—	2.20	—
Hydrocortisone	4.09	2.10	4.50
Triamcinolone	5.04	3.53	—
Desonide	7.25	3.69	6.36
Triamcinolone acetonide	8.59	4.38	7.37

[a] Three or more values were averaged. [b] All compounds except progesterone gave more than one peak due to thermal decomposition. The value given is that of the major peak of each sample.

Samples II and III had to be recrystallized by dissolving the compound in methanol with the aid of steam and allowing the filtered resulting solution to stand in a hood for 3 days before collecting the crystals by filtration. The purities of all compounds were confirmed by HPLC.

Methoxyamine Hydrochloride[9]—This compound was dissolved in pyridine (100 mg/ml) (14) and stored in a conical reaction vial fitted with a polytef-lined valve[10] at 5° in a desiccator.

N-*Trimethylsilylimidazole*[11]—This compound was transferred to a conical reaction vial fitted with a polytef-lined valve immediately after each ampul was opened, and the vial was kept at 5° in a desiccator.

Preparation of Derivatives—*Methoxime–Trimethylsilyl Derivatives*—The procedure of Pfaffenberger and Horning (14) was adapted as follows. The test glucocorticoid (not more than 800 μg) was placed in a 1-ml conical reaction vial fitted with a polytef-lined screw cap[10]. Then 100 μl of methoxyamine hydrochloride–pyridine stock solution was added to dissolve the compound, and the resulting solution was incubated at 70° for 15 min. Then 100 μl of *N*-trimethylsilylimidazole was added, and silylation was conducted at 100° for 10–30 min, depending on the compound tested. An aliquot (1–2 μl) of the final reaction mixture (volume of 200 μl) was injected directly into the gas–liquid chromatograph or GLC–mass spectrometer.

tert-*Butyldimethylsilyl Derivatives*—The procedure was a modified version of previously reported methods (15–18). The test glucocorticoid (not more than 800 μg) was placed in a 1-ml conical reaction vial fitted with a polytef-lined screw cap. Then 100 μl of *tert*-butyldimethylsilyl chloride mixture[12] (1.0 mmole of *tert*-butyldimethylsilyl chloride and 2.5 mmoles of imidazole/ml of anhydrous *N,N*-dimethylformamide) was added to dissolve the compound, and the resulting solution was incubated at 100° for 1 hr. After the reaction, 100 μl of distilled water was added, and the derivatives were extracted into chloroform prior to GLC or GLC–mass spectrometric analysis.

Differential Scanning Calorimetry—A differential scanning calorimeter[8] was employed. All samples were crimped. The rate of temperature increase was 10°/min for each run.

GLC—The recording gas–liquid chromatograph[13] was equipped with a terminal[14] and a flame-ionization detector. The coiled glass column, 1.8 m × 2 mm i.d., contained 3% OV-7 coated onto 80–100-mesh Chromosorb W(HP).

All columns were conditioned at 100° for 15 hr, and then the oven temperature was programmed to increase by 0.1°/min up to 285°. The oven temperature was allowed to remain at 285° for ~15 hr to complete the conditioning process. The flow rate of carrier gas during conditioning was 10 ml/min.

The operating temperatures for routine analysis were: injection port, 265°; column, 285°; and detector, 300°. The gas flow rates were: carrier gas (helium), 60 ml/min; hydrogen, 40 ml/min; and air, 300 ml/min.

GLC–Mass Spectrometry—A computerized gas chromatograph–electron-impact mass spectrometer[15] was used to study the fragmentation pattern of the methoxime–trimethylsilyl derivatives for confirming their chemical structures. The following conditions were used for GLC: injection port temperature, 230°; column temperature, programmed from 200 to 260° at a rate of 10°/min; and carrier gas (helium) flow rate, 20 ml/min. The 2-m × 2-mm i.d. glass column was packed with 3% OV-17 coated onto 80–100-mesh Chromosorb W(HP). For the mass spectra, the ionization beam energy was 80 ev, the source analyzer temperature was 250°, and the separator was at 280°.

The modes of detection included total ion current and selected ion monitoring.

Extraction—The glucocorticoids were double extracted from the cell culture medium[16] containing 10% fetal calf serum as follows. Solid sodium chloride was added to decanted 5–10-ml aliquots of the medium to make it 1 *N*. The aqueous phase was extracted twice with an equal volume of glass-distilled ethyl acetate. After centrifugation to separate the layers, the organic extracts were combined and evaporated to dryness under a gentle nitrogen stream at ambient temperature. The residue was transferred to a 1-ml conical reaction vial containing 100 μl of internal standard (0.50 μg/ml in methanol) by rinsing three times with 0.2 ml of fresh ethyl acetate. The resulting mixture was evaporated to dryness and subjected to derivatization.

A white interface of precipitate occurred in the extraction of glucocorticoids from serum-containing media. The addition of sodium chloride to make a 1 *N* solution avoided this difficulty.

Ethyl acetate, ether, chloroform, and methylene chloride were evaluated for their extraction efficiency of glucocorticoids from serum-free and serum-containing media. Ethyl acetate was chosen because it had a high extraction efficiency and permitted easy withdrawal of the extracts.

Since the presence of serum in the medium decreased the extractability of glucocorticoids by single extraction, double extraction was used.

Recovery Studies—A series of known amounts of the test glucocorticoid solution in methanol (I, III, or IV) was added to individual centrifuge tubes and evaporated to dryness under a nitrogen stream. Five milliliters of 10% serum-containing medium, freshly prepared or decanted from control cells, was added to each glucocorticoid residue. The spiked samples were extracted as already described. Percentage recoveries were calculated from the standard curve of the glucocorticoid that was derivatized without extraction.

Assays on Biological Samples—*Human Dermal Fibroblasts*—Diploid dermal fibroblasts, isolated from explants of biopsies from 25–35-year-old males and stored at −193° in liquid nitrogen, were grown between the fourth and eighth subcultures in monolayer cultures to confluence in plastic culture plates.

Mouse L-929 Dermal Fibroblasts—Aneuploid mouse L-929 fibroblasts[17], originated from a chemically transformed clone in 1943 (19), were used. Both cultured cell lines were maintained in cell culture medium[16] (pH 6.8) supplemented with 10% fetal calf serum, 5 m*M* L-glutamine, 3.7 g of sodium bicarbonate/liter, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μg of amphotericin B[18]/ml. The cells were incubated at 37° in carbon dioxide–air (5:95 v/v). The medium was changed twice weekly. Cellular morphology was examined regularly. Any plate with signs of infection, cellular degeneration, or poor growth was discarded.

Treatment with Glucocorticoids—Stock solutions of the test glucocorticoids (10^3 μg/ml in a propylene glycol vehicle) were prepared by mixing two volumes of propylene glycol (50% in methanol) and one volume of glucocorticoid solution in methanol (10^3 μg/ml) and then removing the methanol under vacuum. A control solution of the propylene glycol vehicle was prepared in a similar fashion.

Desired concentrations of 1 or 10 μg of glucocorticoid/ml of cell culture medium were prepared by adding 1 or 10 μl of the stock solutions/ml to the cell culture medium. The propylene glycol content of these glucocorticoid-treated media did not interfere with cell growth.

The stock solutions as well as the glucocorticoid-containing media were assayed before addition to the cells to ascertain the calculated and actual glucocorticoid concentrations. Some deviations of >15% of expected values were observed because of dilution errors. Only experimentally determined concentrations are reported here.

The glucocorticoid-treated medium was incubated for up to 96 hr with cells that had reached confluence in duplicate culture plates. The decanted media from the two plates containing 6–8 × 10^6 cells were pooled and stored at −4° until analysis. The cells were harvested simultaneously for biochemical studies of glucocorticoid effects on cellular DNA (not reported here).

[9] Lot 04239.19, Pierce Chemical Co., Rockford, Ill.
[10] Reacti-vial with Teflon-lined Mininert valve, Pierce Chemical Co., Rockford, Ill.
[11] Lot 0211774, 10 × 1-g ampuls, Pierce Chemical Co., Rockford, Ill.
[12] Lot 18028, Applied Science Laboratories, State College, Pa.
[13] Model 5880A, Hewlett-Packard, Avondale, Pa.
[14] Model 18850A, Hewlett-Packard, Avondale, Pa.
[15] Varian MAT-111, Varian Associates, Palo Alto, Calif.
[16] Dulbecco Modified Eagle Medium.
[17] Passages 578 and 584, Flow Laboratories, McLean, Va.
[18] Fungizone.

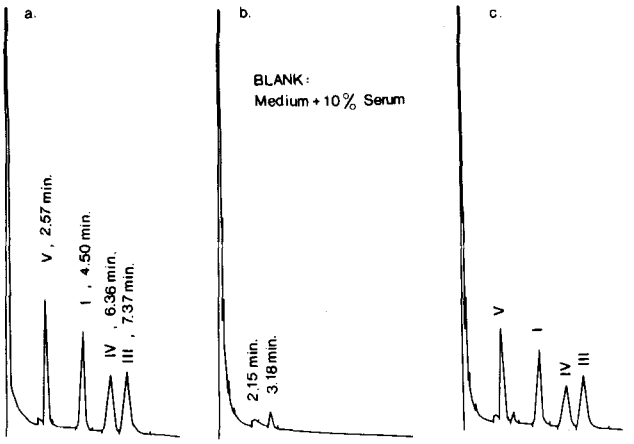


Figure 1—*Typical chromatograms of glucocorticoids and extracts. Key:* a, *extraction from methanol solutions;* b, *extraction from blank medium with 10% serum; and* c, *extraction from serum-containing medium.*

RESULTS AND DISCUSSION

Selection of Derivatizing Agents—For initial experiments, *tert*-butyldimethylsilyl chloride was selected as a potential derivatizing reagent based on the advantages described in the literature (15–18). These advantages are: (*a*) high selectivity of the reaction, by which silylation occurs only at the unhindered C-21 hydroxyl group of the glucocorticoid; (*b*) increased stability of *tert*-butyldimethylsilyl ethers toward hydrolysis, which is 10^4 times higher than that of trimethylsilyl ethers; and (*c*) the simplicity of the mass spectra of *tert*-butyldimethylsilyl ethers because these compounds give intense $(M - 57)^+$ ions. By monitoring this characteristic ion, mass spectrometry has the potential of being a sensitive and selective technique for quantitative analysis of glucocorticoids. The results obtained with this derivative, however, were generally disappointing, and further use of *tert*-butyldimethylsilyl chloride was discontinued. The *tert*-butyldimethylsilyl derivatives had unsatisfactory GLC properties, such as long retention times (longer than those of their parent compounds) and adsorption onto the column due to the remaining free functional groups.

The methoxime–trimethylsilyl derivatives of the test glucocorticoids proved to be superior to their corresponding *tert*-butyldimethylsilyl derivatives based on their satisfactory GLC properties, namely, short retention times and sharp symmetrical peaks with higher responses than their parent compounds.

Column Selection—Columns packed with 3% OV-7, OV-17, and OV-25 were examined for their suitability to resolve peaks of the internal standard, glucocorticoids, and any endogenous component in serum. The OV-7 and OV-17 columns gave superior elution profiles to the OV-25 column. However, with OV-17, the serum-associated endogenous peaks prevented satisfactory resolution of the peak of the internal standard, progesterone, a difficulty not present with the OV-7 column (Figs. 1*b* and 1*c*). Therefore, the OV-7 packing was selected, even though longer retention times were observed (Table I).

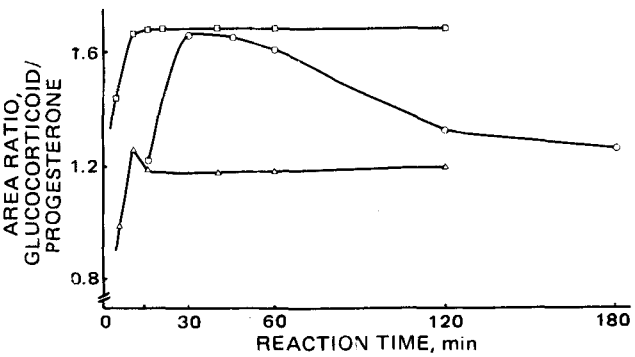


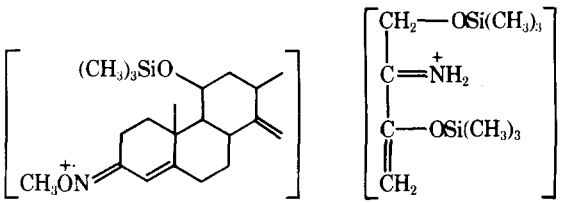
Figure 2—*Reaction kinetics of I–III at 100°. Plot of area ratio of derivatives formed to derivatized internal standard as a function of time. (The concentrations of I–III and their concentration ratios to the internal standard did not have to be the same.) Key:* △, *I;* □, *II; and* ○, *III.*

Optimum Reaction Time for Silylation and Stability of Derivatives—The optimum derivatization time of silylation was evaluated by reacting samples containing equivalent amounts of I–III for various times at 100°. Known amounts of separately derivatized methoxime internal standards then were added. The yield of the derivative, as observed by its area ratio to that of the internal standard, was monitored. The optimum yield was obtained when reaction times were 10, 15, and 30 min for I, II, and III, respectively (Fig. 2).

The methoxime–trimethylsilyl derivatives were stable in excess of 24 hr at 4° when stored in tightly capped reaction vials.

Confirmation of Derivative Formation Using GLC–Mass Spectrometry—The fragmentation patterns of derivatized I–IV when analyzed by electron-impact GLC–mass spectrometry confirmed the formation of methoxime–trimethylsilyl derivatives and V as the methoxime derivative (Figs. 3*a*–3*e*). Little information is available on fragmentation of the methoxime–trimethylsilyl derivatives of the synthetic glucocorticoids. Those of steroids present in rat adrenal gland extracts, primarily corticosterone derivatives, were reported by Prost and Maume (13). Their scheme of characteristic cleavages forms the basis for interpretation of the observed spectra (Figs. 3*a*–3*e*). The well-known losses of *O*-methoxy from *O*-methoxime groups and of silanol from trimethylsilyl groups (13, 20) were observed.

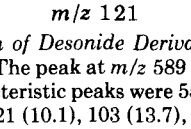
Fragmentation Pattern of Hydrocortisone Derivative, Di(methoxime)-Tri(trimethylsilyl)-I—The peak at *m/z* 636 (1.8%) corresponds to the molecular ion peak; other characteristic peaks were observed at *m/z* 605 (10.5%) $[(M - OCH_3)^+]$, 361 (3.5), 246 (6.1), 147 (7.0) $[(CH_3)_2Si = O^+ - Si(CH_3)_3]$ (21), 103 (11.4) $[CH_2 = O^+ - Si(CH_3)_3]$, 89 (12.3) $[O^+ - Si(CH_3)_3]$, and 73 (100) $[Si^+(CH_3)_3]$.



m/z 361 *m/z* 246

Fragmentation Pattern of Triamcinolone Derivative, Di(methoxime)-Tetra(trimethylsilyl)-II—The peak at *m/z* 739 (0.7%) corresponds to one less than the molecular ion $[(M - 1)^+]$. The characteristic peaks were 709 (3.0%) $[(M - OCH_3)^+]$, 689 (1.4) $[(M - OCH_3 - HF)^+]$, 618 (2.6) $[M - F - (CH_2{-}OSi(CH_3)_3)^+]$, 147 (8.8), 103 (12.3), and 73 (100).

Fragmentation Pattern of Triamcinolone Acetonide Derivative, Mono(methoxime)-Di(trimethylsilyl)-III—The peak at *m/z* 607 (0.4%) corresponds to the molecular ion. The characteristic peaks were 587 (1.1%) $[(M - HF)^+]$, 576 (1.6) $[(M - OCH_3)^+]$, 556 (10.7) $[(M - OCH_3 - HF)^+]$, 121 (9.0), 103 (18.1), and 73 (100).



m/z 121

Fragmentation Pattern of Desonide Derivative, Mono(methoxime)-Di(trimethylsilyl)-IV—The peak at *m/z* 589 (0.8%) corresponds to the molecular ion. The characteristic peaks were 558 (4.9%) $[(M - OCH_3)^+]$, 468 (6.8) $[(M - 121)^+]$, 121 (10.1), 103 (13.7), and 73 (100).

Fragmentation Pattern of Progesterone Derivative, Di(methoxime)-V—The peak at *m/z* 372 (100%) corresponds to the molecular ion. The characteristic peaks were 341 (72.8%) $[(M - OCH_3)^+]$, 286 (29.5), 273 (46.2), 220 (11.5), 153 (57.4), 137 (40.2), 125 (65.6), 100 (75.5), and 87 (39.1).

Resolution of Methoxime–Trimethylsilyl Derivatives on OV-7 and OV-17 Columns—The GLC chromatograms of all four glucocorticoid derivatives appeared as single peaks when eluted from OV-7 columns (Figs. 1*a* and 1*c*). Compounds III and IV, however, resolved into two peaks, a minor peak A with a shorter retention time and occupying <10% of the peak area and a major peak B, when OV-17 packings were used due to the higher degree of resolution obtainable.

A GLC–mass spectrometric examination showed that the chemical structure of the derivative eluting as the minor peak A corresponded to mono(methoxime)–tri(trimethylsilyl)-IV, *m/z* 660, $[(M - 1)^+]$, whereas that of the major peak B corresponded to mono(methoxime)–di(trimethylsilyl)-IV, *m/z* 589 (M^+), as illustrated in Figs. 4*a* and 3*d*, respectively.

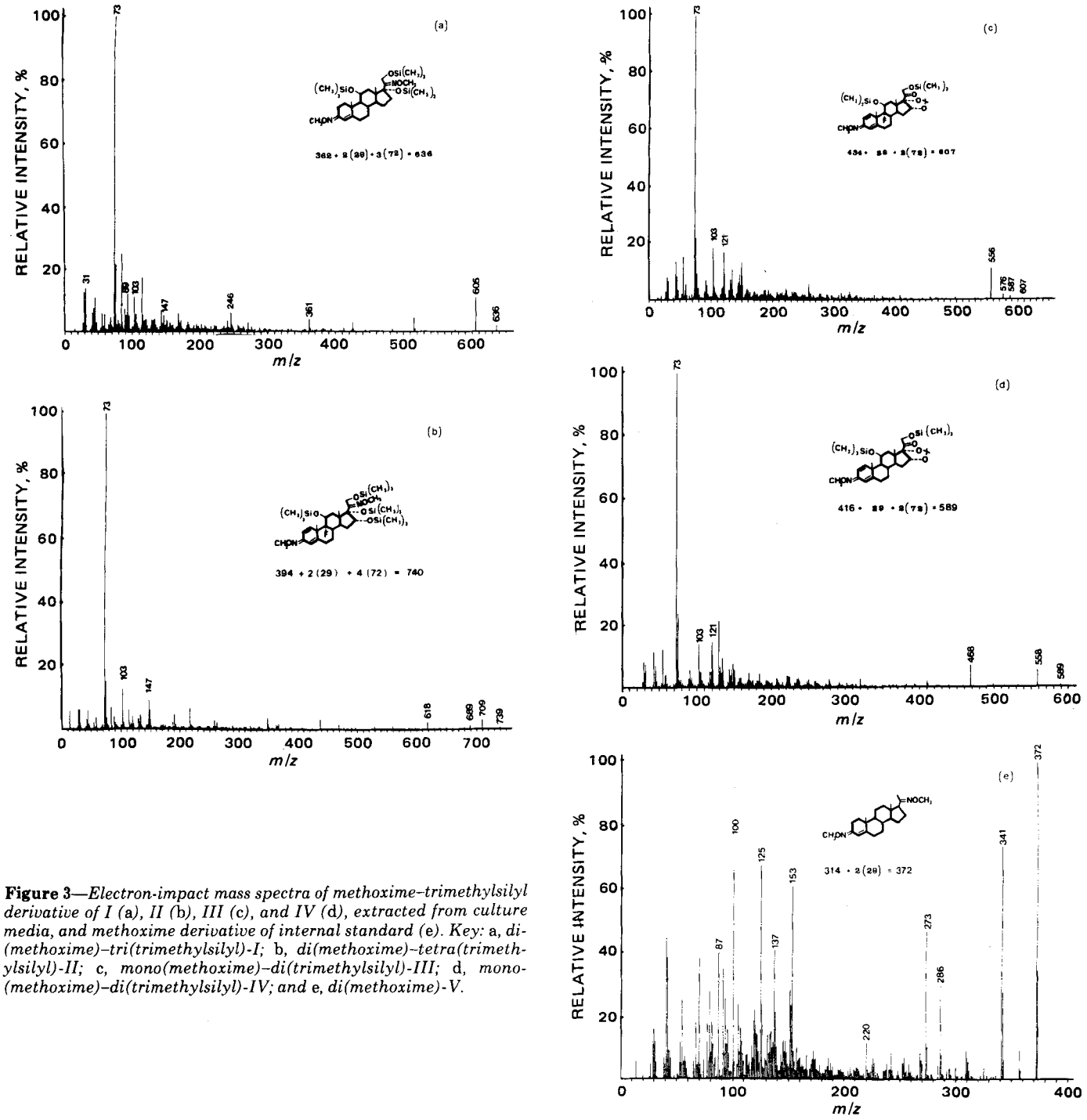


Figure 3—*Electron-impact mass spectra of methoxime–trimethylsilyl derivative of I* (a), *II* (b), *III* (c), *and IV* (d), *extracted from culture media, and methoxime derivative of internal standard* (e). *Key:* a, *di(methoxime)–tri(trimethylsilyl)-I;* b, *di(methoxime)–tetra(trimethylsilyl)-II;* c, *mono(methoxime)–di(trimethylsilyl)-III;* d, *mono(methoxime)–di(trimethylsilyl)-IV; and* e, *di(methoxime)-V.*

Formation of peak A is ascribed as being due to partial enolization of the keto group at C-20. The keto group at C-20 in IV as well as in III, in contrast to I and II, is hindered by the C-16,C-17 acetonide group and remains largely resistant to formation of the methoxime derivative. Under favorable conditions, such as exposure of the reaction mixtures to sunlight radiation and excess silylating reagent, some enolization can occur, which then would lead to the formation of the minor tri(trimethylsilyl) derivative observed.

Indirect evidence for the existence of the enolization reaction was obtained on exposure of the reaction mixture to sunlight. Sunlight is a catalyst for the enolization of various ketones (22). Exposure to sunlight increased the relative magnitude of peak A to peak B, as expected of a sunlight-catalyzed reaction. Removal of the reaction mixture from sunlight reversed the magnitude of peak A to its original levels, indicating the reversibility of sunlight-induced enolization. Prolonging the derivatization time led to an increase in peak A at the expense of the magnitude of peak B, suggesting the formation of A from B.

The fact that the chromatographic peaks from OV-7 columns represent two derivatization products of III and IV, however, did not interfere with the assay because the contributions by the tri(trimethylsilyl) derivatives were <10% of the area of the peaks and were in nearly constant ratio to those of the major di(trimethylsilyl) derivatives. The derivatization time should be kept at 30 min, and light should be excluded.

Compound III shared all of the previously mentioned characteristics of derivatization and gave mono(methoxime)–di(trimethylsilyl)-III, *m/z* 607 (M^+), as the major product and mono(methoxime)–tri(trimethylsilyl)-III, *m/z* 679 (M^+), as the minor product (Figs. 3*c* and 4*b*).

The presence of antibiotics in the medium showed no interference on the chromatograms.

Calibration Curves—Calibration curves were constructed for I, III, and IV extracted from medium containing 10% serum. The curves were linear within the ranges studied, namely, 40–1600 ng/μl of derivatized solution for I (equivalent to 1.6–65.8 μg/ml of medium), 60–1600 ng/μl of derivatized solution for III (equivalent to 2.4–63.7 μg/ml of medium),

Table II—Estimation of I after Extraction from Spiked Medium Samples (n = 3)

Amount Added to 5 ml of Serum-Containing Medium, μg	Weight Ratio[a]	Mean Peak Area Ratio[b] ± SD	Amount Recovered[c], μg	Mean Recovery ± SD, %
Mar. 1978				
16.45	0.1347	0.1046 ± 0.0095	13.95	84.82 ± 6.86
24.67	0.2025	0.1444 ± 0.0021	18.69	75.75 ± 1.00
41.42	0.3372	0.2804 ± 0.0202	34.86	84.77 ± 5.84
61.68	0.5058	0.4393 ± 0.0281	53.75	87.15 ± 5.36
164.48	1.3480	1.1930 ± 0.0518	143.38	87.17 ± 3.71
Aug. 1978				
8.22	0.0667	0.0569 ± 0.0010	7.92	97.51 ± 5.52
16.45	0.1335	0.1406 ± 0.0076	18.39	111.82 ± 5.54
24.67	0.2003	0.1804 ± 0.0013	21.65	90.16 ± 4.39
41.12	0.3338	0.3550 ± 0.0222	44.11	107.28 ± 6.48
61.68	0.5006	0.4920 ± 0 0238	60.55	98.17 ± 4.63
82.24	0.6676	0.6486 ± 0.0211	79.34	96.47 ± 3.08
164.48	1.3351	1.3121 ± 0.0195	158.93	96.63 ± 1.42
328.96	2.6701	2.5330 ± 0.0465	305.40	92.84 ± 1.69

[a] Weight ratio of I/V (internal standard). [b] Peak area ratio of derivatized I/derivatized V. Mean values of triplicate samples, each determined by three measurements. [c] Calculated from the standard curve of I without extraction, $y = mx + c$, where $m = 1.026$ and $c = -0.009$ ($r^2 = 0.999$), and $y = mx + c$, where $m = 0.947$ and $c = -0.005$ ($r^2 = 0.998$) for extracted samples, as plotted for the mean area ratio *versus* weight ratio. The average mean recovery was 93.12 ± 9.74%.

and 20–300 ng/μl of derivatized solution for IV (equivalent to 0.80–12.0 μg/ml of medium) (Tables II–IV).

Two calibration curves of I were determined with a time lapse of 6 months between them. The relative standard deviation in slope values was 3.1%. The combined data gave a straight line of $y = 0.947x - 0.005$ with $r^2 = 0.998$, indicating satisfactory assay reproducibility.

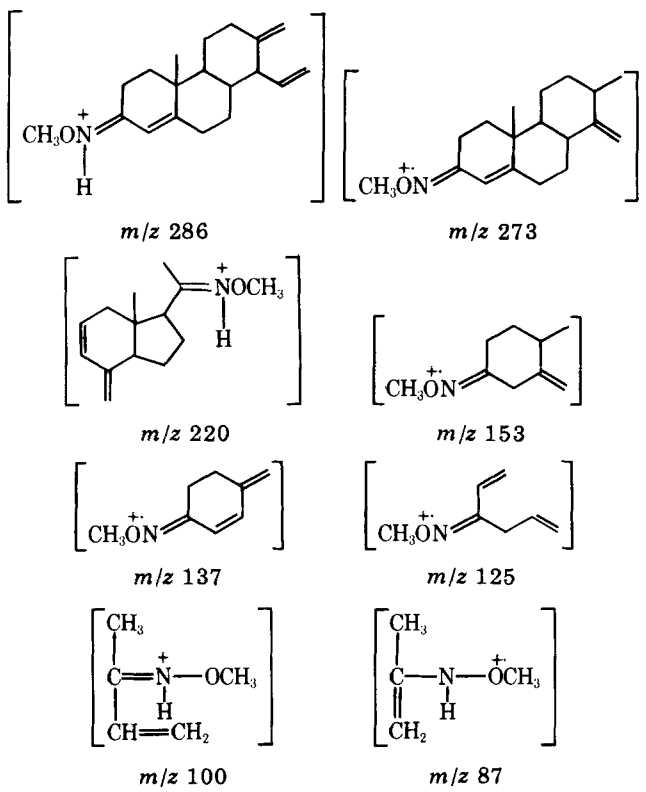
Three calibration curves of III were determined within 1 year. The relative standard deviation of slope values was 7.6%. The combined data gave a straight line of $y = 0.553x + 0.012$ with $r^2 = 0.996$. The satisfactory reproducibility of the assay confirmed the validity of using the sum of double-peak areas, mono(methoxime)–di(trimethylsilyl)-III and mono(methoxime)–tri(trimethylsilyl)-III, based on the assumption that the yield ratio of these two peaks was a constant.

The recoveries of three glucocorticoids added in various amounts also were tabulated (Tables II–IV). The average mean recoveries were 93.12 ± 9.74, 85.64 ± 7.19, and 97.73 ± 4.72% for I, III, and IV, respectively.

Glucocorticoids generally are used in cell cultures at concentrations smaller than those used for preparing the calibration curves. However, these lower concentrations can be compensated readily by extracting >5 ml of the medium employed in the preparation of the calibration curves. On the other hand, the amounts used are realistic for assays of stock solution concentrates and similar solutions of pharmaceutical interest.

Biological Data—The applicability of the assay was demonstrated by determining the I, III, and IV levels in serum-containing cell culture medium after various incubation times with cultured human or mouse dermal fibroblasts (Tables V–VII).

The time profile of III indicated that its prolonged incubation of up



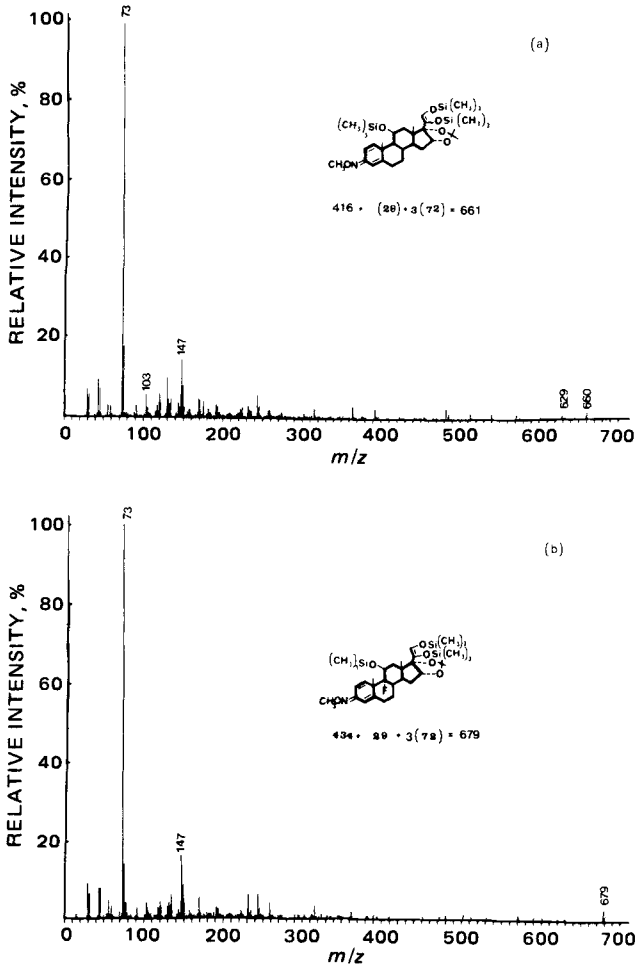


Figure 4—*Electron-impact mass spectra of mono(methoxime)–tri(trimethylsilyl)-IV* (a) *and mono(methoxime)–tri(trimethylsilyl)-III* (b).

Table III—Estimation of III after Extraction from Spiked Medium Samples (n = 3)

Amount Added to 5 ml of Serum-Containing Medium, μg	Weight Ratio[a]	Mean Peak Area Ratio[b] ± SD	Amount Recovered[c], μg	Mean Recovery ± SD, %
Apr. 1978				
25.92	0.1420	0.0725 ± 0.0125	22.75	87.75 ± 11.09
39.00	0.2137	0.1228 ± 0.0220	34.95	89.62 ± 12.36
64.80	0.3541	0.2142 ± 0.0089	57.13	88.17 ± 3.33
86.40	0.5314	0.2919 ± 0.0219	75.99	87.96 ± 6.15
129.13	0.7081	0.4082 ± 0.0311	104.23	80.65 ± 4.37
257.70	1.4120	0.8049 ± 0.0354	200.53	77.82 ± 2.49
Oct. 1978				
11.94	0.0969	0.0508 ± 0.0031	11.79	98.76 ± 4.25
15.92	0.1293	0.0694 ± 0.0012	14.84	93.21 ± 1.24
23.88	0.1938	0.1030 ± 0.0114	20.35	85.20 ± 7.82
39.80	0.3231	0.1778 ± 0.0001	32.60	81.92 ± 0.20
59.70	0.4846	0.2777 ± 0.0076	48.98	82.04 ± 2.08
79.60	0.6462	0.3628 ± 0.0053	62.92	79.05 ± 1.09
159.20	1.2922	0.7495 ± 0.0282	126.29	79.33 ± 2.90
318.4	2.5840	1.5506 ± 0.0356	257.58	80.90 ± 1.83
Feb. 1979				
11.94	0.1189	0.0659 ± 0.0040	11.63	97.37 ± 4.90
15.92	0.1586	0.0932 ± 0.0059	15.27	95.93 ± 4.95
23.88	0.2378	0.1435 ± 0.0111	21.99	92.08 ± 6.21
39.80	0.3964	0.2330 ± 0.0027	33.94	85.28 ± 0.91
59.70	0.5946	0.3556 ± 0.0253	50.32	84.28 ± 5.66
79.60	0.7928	0.5082 ± 0.0349	70.69	88.81 ± 5.86
159.2	1.5860	0.9127 ± 0.0962	124.72	78.34 ± 8.07
318.4	3.1710	1.6403 ± 0.0042	221.88	69.69 ± 0.17

[a] Weight ratio of III/V (internal standard). [b] Peak area ratio of derivatized III/derivatized V. Mean values of triplicate samples, each determined by three measurements. [c] Calculated from the standard curve of III without extraction, $y = mx + c$, where $m = 0.750$ and $c = -0.021$ ($r^2 = 0.990$), and $y = mx + c$, where $m = 0.553$ and $c = 0.012$ ($r^2 = 0.996$) for extracted samples, as plotted for the mean area ratio *versus* weight ratio. The average mean recovery was 85.64 ± 7.19%.

Table IV—Estimation of IV after Extraction from Spiked Medium Samples (n = 3)

Amount Added to 5 ml of Serum-Containing Medium, μg	Weight Ratio[a]	Mean Peak Area Ratio[b] ± SD	Amount Recovered[c], μg	Mean Recovery ± SD, %
4.0	0.0787	0.0395 ± 0.0030	3.95	98.77 ± 4.72
8.0	0.1625	0.1063 ± 0.0060	8.15	101.97 ± 4.70
12.0	0.2415	0.1693 ± 0.0057	12.12	101.03 ± 3.01
16.0	0.3252	0.2360 ± 0.0046	16.32	102.03 ± 1.81
24.0	0.4681	0.3500 ± 0.0078	23.49	97.91 ± 2.05
40.0	0.7254	0.5551 ± 0.0256	36.42	91.04 ± 4.03
60.0	1.0919	0.8473 ± 0.0907	54.81	91.36 ± 9.53

[a] Weight ratio of IV/V (internal standard). [b] Peak area ratio of derivatized IV/derivatized V. Mean values of triplicate samples, each determined by three measurements. [c] Calculated from the standard curve of IV without extraction, $y = mx + c$, where $m = 0.790$ and $c = -0.023$ ($r^2 = 0.999$), and $y = mx + c$, where $m = 0.713$ and $c = -0.0038$ ($r^2 = 0.999$) for extracted samples, as plotted for the mean area ratio *versus* weight ratio. The average mean recovery was 97.73 ± 4.72%.

Table V—Levels of I in Media as a Function of Time after Incubation with Cultured Human Dermal Fibroblasts

Incubation Time, hr	n	Concentration Assayed, μg/ml of medium	
		A-2[a] (Percent Remaining Intact)	A-4[b]
0	2	0.7307 ± 0.1338 (100.00 ± 18.31)[c]	—
0.25	1	—	0.4621
2	2	—	0.3921 ± 0.0045
4	2	0.3892 ± 0.0345 (53.26 ± 4.72)	0.5194 ± 0.0091
6	2	—	0.4737 ± 0.0091
8	2	—	0.4336 ± 0.0156
12	2	—	0.4364 ± 0.0130
24	2	0.3864 ± 0.0441 (52.88 ± 6.04)	0.3464 (n = 1)
48	2	—	0.4545 ± 0.0497

[a] Human dermal fibroblasts, F.H. passage 6. [b] Human dermal fibroblasts, W.P. passage 4. [c] The concentration of I recovered from medium containing no cells was defined as 100% of intact drug.

to 83.5 hr with cultured mouse L-929 dermal fibroblasts did not decrease intact III in media. About 20% of III was removed from the culture medium by absorption and/or adsorption by cells 2 hr after administration of III. The levels of III at various incubation periods between 2 and 83.5 hr did not vary appreciably. A significant decrease of III after 94 hr was observed but unexplained.

To eliminate the possibility of decomposition, the stability of III in the medium upon incubation was studied at 37° in the absence of cells for 72 and 96 hr. There was no appreciable decomposition due to the incubation at 37°.

The levels of IV after 4 and 24 hr of incubation with mouse L-929 fibroblasts indicated ~20% loss of IV from the medium.

Therefore, for any biological effect of synthetic glucocorticoids observed at the dose level of 1 μg/ml of medium, the true bioavailable dose was probably not more than 0.2 μg/ml of medium.

The levels of I as a function of time were measured twice at various incubation intervals. The loss of I from the media due to cells was larger than that of the synthetic glucocorticoids. Compound I had rather constant levels when incubated between 0.25 and 48 hr.

Metabolites—No extra peaks were observed in samples incubated

Table VI—Levels of III in Media as a Function of Time after Incubation with Cultured Mouse L-929 Dermal Fibroblasts

Incubation Time, hr	n	Concentration Assayed, μg/ml of medium	Intact III Remaining in Media, %
0	5	1.265 ● 0.0820	100.00 ± 6.48[a]
2	2	1.032 ± 0.0029	81.58 ± 0.23
4	2	1.025 ± 0.0007	81.01 ± 0.06
8	2	1.030 ± 0.0101	81.45 ± 0.80
11	2	1.011 ± 0.0045	79.94 ± 0.36
24	2	1.060 ± 0.0050	83.77 ± 0.40
48	1	1.011	79.94
72	2	1.028 ± 0.0018	81.25 ± 0.14
83.5	2	1.079 ± 0.0300	85.29 ± 2.37
94	2	0.919 ± 0.022	72.67 ± 1.74

[a] The concentration of III recovered from medium containing no cells was defined as 100% of intact drug.

with cells for 0–108 hr, and there was no appreciable change in the magnitudes of endogenous peaks in the mixture due to the medium and 10% serum. It was proposed that no metabolite was apparently present unless its methoxime–trimethylsilyl derivative had the same retention time as the derivatized parent compound or that the amounts of metabolites were too small to be detected.

Applicability of Methoxime–Trimethylsilyl Reaction to Other Glucocorticoids—Another glucocorticoid, diflorasone diacetate, was derivatized under the same conditions. However, the reaction product gave four peaks in the gas–liquid chromatogram, indicating multiple derivatizing products, presumably due to the hydrolysis of acetates by hydrochloride generated in the reaction with methoxyamine hydrochloride and subsequent silylations at the C-17 and/or C-21 free hydroxyl groups. Therefore, diflorasone diacetate and probably other ester-containing glucocorticoids are not amenable to GLC analysis using the subsequent derivatization with methoxyamine hydrochloride and *N*-trimethylsilylimidazole.

Prednisolone could be derivatized to yield the di(methoxime)–tri(trimethylsilyl)prednisolone derivative with the same reaction procedure.

Table VII—Levels of IV in Media as a Function of Time after Incubation with Cultured Mouse L-929 Dermal Fibroblasts

Incubation Time, hr	n	Concentration Assayed, μg/ml of medium	Intact IV Remaining in Media, %
0	4	0.6073 ± 0.0120	100.00 ± 1.98[a]
4	2	0.5136 ± 0.1006	84.58 ± 11.70
24	2	0.4964 ± 0.0326	81.74 ± 5.37

[a] The concentration of IV recovered from medium containing no cells was defined as 100% of intact drug.

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